BACTERIOLOGICAL REVIEWS, Mar., 1972, p. 65-108
Vol. 36, No. 1

Printed in U.S.A.

Intermediary Metabolism of Mycobacteria

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INTRODUCTION

The last comprehensive review devoted to this topic was made in 1951 by Edson (81), and the present review is designed to cover work done after that year. Since that time, however, two special reviews have appeared, one on biochemical properties of virulent and avirulent strains of *Mycobacterium tuberculosis* by Bloch (37) in 1960 and the other on the enzyme systems in saprophytic and avirulent strains of mycobacteria by Goldman (113) in 1961. Metabolism in experimental tuberculosis and the structure of the cell wall and immunological aspects of mycobacteria are not included, because, although a wealth of information has recently accumulated in these areas, they do not legitimately come within the scope of this review.

The genus *Mycobacterium* encompasses a variety of organisms ranging from such pathogenic species as *M. tuberculosis* and *M. paratuberculosis* and their mutants, avirulent, drug-resistant, etc., to the apparently nonpathogenic species such as *M. phlei*, *M. smegmatis*, and *M. lacticola*. The common properties linking these organisms include the similarity of the basic structure of peptidoglycans, mycosides, and mycolic acids of their cell walls, the degree of homology found among their deoxyribonucleic acids, the limited auxotrophy of most of them, and, with rare exception, their acid-fastness. Only about half a dozen laboratories in the world have been studying the metabolism of the pathogenic typical mycobacteria, and this is all the more surprising since tuberculosis is generally conceded to be today the most important communicable disease in the world. Hence, for the development of effective antimicrobials, basic data on the metabolism of the organism responsible is most important.

The three species of the genus *Mycobacterium* concerned in the tuberculosis of mammals and birds are *M. tuberculosis*, *M. bovis*, and *M. avium*, known as the human, bovine, and avian strains, respectively, and denoting chiefly the relative distribution of the bacterium in the respective hosts. The role of the "atypical" mycobacteria, such as *M. intracellulaire*, *M. kansasii*, and *M. scrofulaceum*, in the spectrum of chest diseases is extremely important. The problem of pulmonary infection with atypical acid-fast mycobacteria has been receiving considerable attention of late, since the number of reports of well-documented cases of pulmonary disease, apparently caused by atypical mycobacteria, has been steadily increasing for many years. In general, they exhibit poor susceptibility to antimicrobial drugs, and many of them produce pigments during growth. Except for a report (99) on determination of dehydrogenase, practically no information is available on the metabolism of pathogenic atypical mycobacteria.

Regarding *M. leprae*, various laboratories are still trying to standardize growth conditions for this bacterium in a laboratory medium, so that, if this is successful, biochemical tech-

niques can be applied to unravel the mystery of this organism. In recent years, however, there has been partial success in growing some strains in a defined medium. Available metabolic information on this bacterium will be cited at appropriate places in this review.

A section is devoted to the mode of action of the currently available antitubercular drugs because of its importance in both fundamental and applied aspects. And, since the biochemist cannot ignore our developing knowledge of genetics, the genetic aspects of mycobacteria have been dealt with briefly. For a fuller review of this aspect, reference is made here to a convenient summary of knowledge emerging from the recent symposium edited by Juhasz and Plummer (181) on host-virus relationships in mycobacteria, nocardia, and actinomycetes.

GROWTH OF MYCOBACTERIA

One important reason that progress in the work on the metabolism of *M. tuberculosis* has been tardy is the slow growth of the organism on laboratory media; several investigators have attempted to devise better media for its growth. Some of the conditions for increasing the rate of growth of *M. tuberculosis* are the use of shake culture, an increase in the size of inoculum, substitution of glutamate for asparagine as nitrogen source, and use of glycerol as carbon source (44). A few growth factors have also been reported for the slow-growing species of mycobacteria. Yamane (425) has isolated from egg-yolk a crystalline growth-enhancing factor which is heat-stable and is required in concentrations of 0.01% and above. Ramakrishnan, Indira, and Sirsi (298, 299) reported the isolation of a heat-stable polysaccharide growth-promoting factor for *M. tuberculosis* from coconut water; this substance has antigenic similarity to a component in the bacillus (249). Better known are the iron-chelating growth factors, mycobactins from mycobacteria themselves—a topic recently covered in a extensive review by Snow (336). Mycobactins promote the growth of *M. paratuberculosis* and are unique in the sense that they are highly specific within a bacterial group. A recent report (155) that L-asparagine (which is commonly used as the nitrogen source for the growth of *M. tuberculosis*) contains an isoflavanoid as impurity and that the stimulatory properties ascribed to L-asparagine may be due to this isoflavanoid is interesting and should encourage further work on the effect of various isoflavanoids on the growth of slow-growing mycobacteria.

CARBOHYDRATE METABOLISM

Assimilation of Glycerol and Glucose

Glycerol is the primary carbon source employed in the culture of mycobacteria, though the organisms can also use glucose. *M. phlei* cells grown on glycerol medium and glucose medium have identical rates of growth as judged by protein and deoxyribonucleic acid (DNA) synthesis, but glycerol-grown cells have, per unit volume of medium, consistently a greater weight than glucose-grown cells (364). The increased weight of glycerol-grown cells is attributable to an increased lipid and polysaccharide content. There is also a basic difference in the kinetics of uptake and utilization of glucose and glycerol by *M. phlei* (365). With glycerol, the rates of uptake, respiration, and assimilation are saturated at low substrate concentration, whereas with glucose they do not show saturation even at high concentrations. These quantitative differences in the utilization of glycerol and glucose can account for the increased content of lipid and polysaccharide found in glycerol-grown *M. phlei* and probably also in *M. tuberculosis*. By employing ^{14}C-labeled glycerol and analyzing the distribution of the label, Edson et al., (82) concluded that, in *M. butyricum*, glycerol is degraded to α-ketoglutarate and carbon dioxide through pyruvate. The presence of glycerol 3-phosphate dehydrogenase has been demonstrated in *M. butyricum* (156) and in *M. bovis* BCG (406). Further, 8-day-old cultures of the latter organism grown on a rotary shaker do not possess any glycerol dehydrogenase activity. The phosphorylation of glycerol followed by dehydrogenation is therefore considered to be the main route of glycerol metabolism in *M. bovis* BCG. Indira and Ramakrishnan demonstrated the presence of a nicotinamide adenine dinucleotide phosphate (NADP)-dependent glycerol 3-phosphate dehydrogenase and glycerol dehydrogenase in a 14-day culture of *M. tuberculosis* H37Ra (160) and a nicotinamide adenine dinucleotide (NAD)-dependent glycerol 3-phosphate dehydrogenase in *M. tuberculosis* H37Rv (161). It is possible that the appearance of glycerol dehydrogenase activity in the former experiments is due to the low oxygen tension, as has been reported for *Streptococcus faecalis* (166). An NADP-dependent glycerol 3-phosphate dehydrogenase has also been noted in *M. smegmatis* (326).

Glycolytic and Oxidative Pathways

The glyceraldehyde 3-phosphate formed

from glycerol is apparently metabolized further by conventional pathways. Qualitatively these pathways in the virulent and avirulent strains appear to be identical.

Evidence for a functional glycolytic system in the *M. tuberculosis* H37Ra has been presented (31), and the key enzymes of both the glycolytic and the hexose monophosphate shunt (HMP) pathways have been shown to be present in the glucose-grown cells of H37Ra strain (160, 161) and in the H37Rv strain (162, 354). Le Cam, Madec, and Bernard (224), however, could not detect glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase in glycerol-grown cells of *M. tuberculosis*, H37Ra, and they suggested that in these organisms glycolysis is predominant over the HMP pathway. In glycerol-grown cells of *M. phlei*, however, the enzymes of both pathways could be detected. The presence of an inhibitor in extracts of H37Ra strain which masks the activity of phosphofructokinase activity has also been reported (31).

Cell-free extracts of *M. tuberculosis* H37Rv, BCG, and *M. avium* exhibit aldolase activity (19). The enzyme activity is maximal in the extracts of H37 Rv, and the optimum *p*H is 8.6 in the case of all the strains. Co^{2+}, Fe^{2+}, Zn^{2+}, Ni^{2+}, and Mn^{2+} as well as metal chelators inhibit the enzyme activity. Since the organisms grow well in the presence of Fe^{2+}, Zn^{2+}, and Mn^{2+}, the significance of their inhibition of aldolase is not clear. The individual enzymes of the glycolytic pathway and shunt in mycobacteria have not been extensively purified.

The effect of age of the culture on the activities of some enzymes of glycolysis and hexose-monophosphate pathways has been studied in *M. phlei* and *M. tuberculosis* H37Ra (224) and in *M. smegmatis* (326). In *M. smegmatis*, the activity of all the enzymes with the exception of glycerol 3-phosphate dehydrogenase decreases from the second day to the sixth day and tends to increase again from the eighth day. In *M. phlei* and *M. tuberculosis*, however, the activities show an irregular pattern.

The metabolism of the end product of glycolysis, i.e., lactate, has been worked out in detail in mycobacteria. The presence of lactate oxidative decarboxylase (which oxidizes lactate to acetate, carbon dioxide, and water) has been reported in *M. smegmatis*, *M. phlei*, and *M. stercoris* and in *M. tuberculosis* H37Rv (67). This enzyme has been crystallized from *M. phlei* (357) and *M. smegmatis* (344). The enzyme has a molecular weight of 30,000 to 40,000, contains 6 to 8 moles of flavine mononucleotide (FMN) per mole of protein as its prosthetic group, and catalyzes the oxidation of only L-lactate and α-hydroxy-*n*-butyrate. It is inhibited competitively by D-lactate.

Recent years have been marked by efforts to use relatively simple isotope techniques to determine the balance of alternative respiratory pathways. To discover whether the inability of the avirulent strain to multiply in tissues, where generally low oxygen tension prevails, is attributable to its more predominantly oxidative metabolic pathways, Ramakrishnan, Indira, and Maller (300) studied the oxidation of glucose by *M. tuberculosis* H37Rv and H37Ra strains through the labeling of CO_2 formed from uniformly labeled glucose as well as glucose-*1*-^{14}C and glucose-6-^{14}C. The glucose dissimilation in the avirulent strain takes place to an equal extent via the glycolytic and oxidative pathways, whereas in the avirulent strain, the dissimilation is predominantly via the glycolytic pathway, which accounts for 85% of the glucose utilized. By using essentially similar methods, O'Barr and Rothlauf (271) estimated that about 97% of glucose is metabolized by drug-susceptible *M. tuberculosis* through the glycolytic pathway, whereas strains resistant to streptomycin, *p*-aminosalicylic acid, and isoniazid metabolize glucose through this pathway to a slightly smaller extent. More detailed studies (159), also using glucose-2-^{14}C and analysis by chemical degradation of the pyruvate formed, showed that, even in the avirulent strain, the glycolytic pathway dominates to the extent of about 74% of the glucose metabolized. It must be admitted that, based on the work of Katz and Wood (187), the methods used in the earlier-mentioned studies are not free from errors in interpretation.

Metabolism of Other Sugars

Although mycobacteria have been reported to prefer glucose or glycerol over other carbohydrates as carbon source, they apparently possess enzymes to metabolize the latter. Kimura and Sasakawa (195) reported that an enzyme preparation from *M. avium* with polyol dehydrogenase activity utilized myo-inositol, D-mannitol, glycerol, propanediol-1,2, and ethylene glycol in the presence of NAD, but it is not clear whether this enzyme is identical with glycerol- or glycerol 3-phosphate dehydrogenase. Hey-Ferguson and Elbein (148) found that *M. smegmatis* grows much more rapidly on mannose and fructose than on glucose and that cells grown on these sugars have an enzyme, D-mannose ketol isomerase. The enzyme has been purified 60-fold; it catalyzes the conversion of D-mannose to D-lyxose and fructose.

The occurrence of a β-D-galactosidase has been reported in *M. smegmatis* (167). A transglucosylase which catalyzes the synthesis of trehalose 6-phosphate from glucose 6-phosphate and uridine diphosphate-glucose has been studied in *M. tuberculosis* H37Rv and H37Ra (236). The avirulent strain H37Ra contains also an inhibitor which acts on the enzyme of both the avirulent and virulent strains. The inhibition is noncompetitive. The inhibitor is an oligoribonucleotide containing between six and nine purine bases but no pyrimidine bases and has been given the trivial name of mycoribnin. During purification, the transglucosylase loses its sensitivity to mycoribnin but gains sensitivity if the bicarbonate concentration of the preparation is reduced to about 0.01 mM; bicarbonate and mycoribnin are probably bound to the same site on the enzyme (237). The transglucosylase from brewer's yeast when prepared free from bicarbonate is also inhibited by mycoribnin.

Lornitzo and Goldman (238, 239) have studied the occurrence in *M. tuberculosis* H37Ra of a number of related polysaccharides containing 6-*O*-methyl D-glucose, as they may act as precursors to polysaccharides containing 6-*O*-methyl D-glucose and other methylated sugars (239). Cell-free extracts of the organism contain an enzyme which catalyzes the transfer of the methyl group of *S*-adenosylmethionine to α-glycerol phosphate, forming 3-methoxy 1,2-propanediol (α-glyceryl methyl ether). The methoxy group is thus introduced at the triose level for incorporation into hexoses and other sugars.

A new type of kinase—inorganic polyphosphate: D-glucose 6-phosphotransferase—has been reported in *M. phlei* (355). The enzyme has been partially purified and some of its properties have been studied; however, its role in the overall metabolism of glucose, if any, has not been worked out.

Endogenous Metabolism

The endogenous metabolism of *M. phlei* has been linked to the presence of glucose-6-phosphate dehydrogenase functioning with reduced NADP (NADPH)-2,6-dichlorophenol-indophenol reductase (353). The necessary substrate and coenzyme, i.e., glucose-6-phosphate and NADP, were found to be contained in the cell-free extract, and the only required addition to activate the system is a suitable electron aceptor. The substrates for endogenous metabolism are identified as the phosphate esters of the glycolytic pathway, energy-rich phosphates, and nucleotides in *M. smegmatis* (306). The endogenous respiration in *M. tuberculosis* is stimulated by uncoupling agents like dinitrophenol and azide, and inhibited by sulfhydryl binding reagents and arsenite, indicating that the stored endogenous substrates are utilized via pathways similar to glycolysis and the tricarboxylic acid cycle (351). Further work on the characterization of the endogenous substrates, especially in *M. tuberculosis*, would be highly desirable.

Tricarboxylic Acid Cycle

The first evidence for the occurrence of the tricarboxylic acid cycle in mycobacteria was provided by Youmans, Millman, and Youmans (430), who showed that acetone-dried cells of *M. tuberculosis* H37Rv oxidize all the intermediates of the cycle. Goldman (106–111) has partially purified isocitric dehydrogenase, malic dehydrogenase, condensing enzyme (108), pyruvic oxidase, and pyruvic dehydrogenase system from *M. tuberculosis* H37Ra. For details, the reader is referred to the review by Goldman (113). The individual enzymes of the tricarboxylic acid cycle and the glyoxylate bypass are present in sonic extracts of the H37Rv strain, and, except for malate dehydrogenase, all the other dehydrogenases investigated are NADP- but not NAD-dependent (350). A powerful reduced NAD (NADH) oxidase (121, 161, 350) present in the extracts prevents even the NAD reduction unless it is carried out at a *p*H unfavorable to the activity of the oxidase. It is probable that the presence of NADP-requiring dehydrogenases in *M. tuberculosis* guarantees a constant high concentration of NADPH as a reducing agent for its metabolic processes, especially for the biosynthesis of lipids, and the presence of NADH oxidase in the organism ensures that sufficient NAD is available as an oxidizing agent in these processes. In *M. tuberculosis* H37Rv, NAD is present in double the concentration of NADP, whereas the reverse is true for the corresponding reduced compounds (121). The NAD content of H37Ra is similar to the H37Rv strain, but NADP, NADH, and NADPH are present in almost equal proportions in H37Ra (223). *M. tuberculosis* appears to be similar to *M. phlei* with respect to its pyridine nucleotide coenzyme content (399) in that both contain NAD and NADP, unlike *M. butyricum* which contains only NAD in the oxidized form (183). The ratio of NAD to NADP is 6.0 in *M. phlei* and 2 to 3 in *M. tuberculosis* (121, 223).

The operation of the glyoxylate cycle in cell-free extracts of *M. tuberculosis* H37Ra has also been demonstrated, and the enzymes of the

cycle, viz., isocitrate lyase and malate synthetase, have been purified (117). A glycine dehydrogenase catalyzing the reductive amination of glyoxylate to glycine has also been purified from this strain. The specific activity of all the enzymes of the tricarboxylic acid cycle increases with age of the culture up to 14 days and decreases after 21 days; however, the activity of isocitrate lyase increases continuously with age up to 28 days (265).

In addition to those already reported for *M. tuberculosis* H37Ra, some of the individual enzymes of the tricarboxylic acid cycle have been purified from other species of mycobacteria. The occurrence of malate dehydrogenase and malic enzyme in different species of mycobacteria has been investigated by Parvin Khan, Pande, and Venkatasubramanian (282). NAD-dependent malate dehydrogenase is high in *M. phlei*, considerably less in *M. tuberculosis* H37Rv, and not at all detectable in *M. smegmatis* 607. Although NAD-dependent malate dehydrogenase is not detectable in another strain of *M. smegmatis*, NADP-dependent malate dehydrogenase is present in both the strains (326). On the other hand, malic enzyme, which is present in all the species of mycobacteria studied, is highest in *M. smegmatis* 607; this enzyme has been purified 100-fold (283). The enzyme requires NADP and bivalent metal ions like Mn^{2+} or Mg^{2+} for activity and is inhibited by sulfhydryl binding agents. Substrate concentrations above 2.5 mM inhibit the enzyme, but this inhibition can be reversed by increasing the Mg^{2+} concentration. Kimura and Tobari (196, 370) purified malate dehydrogenase from *M. avium* and showed that the enzyme requires flavine adenine dinucleotide (FAD) and phospholipid for its activity; phospholipid is apparently necessary to make this particulate enzyme active.

The intact cells of *M. tuberculosis* H37Rv grown in vitro on laboratory media and in vivo in the lungs of infected mice show succinic dehydrogenase activity, but not succinoxidase activity (322). On the other hand, extracts of only the in vitro-grown bacilli have succinoxidase activity. Since permeability for succinate is apparently not a problem for these organisms, the conclusion drawn was that, in the pathogenic state, *M. tuberculosis* as present in tuberculous animals is deficient in certain components of the terminal respiratory system. A confirmation of this thesis by a systematic analysis of the electron transport system in this strain would be most interesting. Bekierkunst and Artman reported (34) that cell-free extracts of *M. tuberculosis* H37Rv grown in the lungs inhibited lactic dehydrogenase of BCG extracts and succinoxidase activity of lung tissues, in agreement with the earlier reports (323, 324) that the properties of lung-grown organisms are different in some respects from those grown on laboratory media. However, the nature of the inhibitor in lung-grown tubercle bacilli has been established and identified as nicotinamide adenine dinuclease activity (20).

Resting cells of mycobacteria grown on glucose or glycerol do not oxidize most of the intermediates because of permeability barrier. The intact cells of *M. smegmatis* grown on fumarate and acetate oxidize these intermediates rapidly, and the organism may possess permeases for fumarate and acetate (83). However, no direct evidence for any permease has been reported in mycobacteria.

The fixation of carbon dioxide into malonate by *M. avium* has been demonstrated (218); the enzyme, fractionated with ammonium sulfate, requires coenzyme A and Mn^{2+} for activity.

LIPID METABOLISM

The chemistry of the lipid constituents and the lipid composition of different species of mycobacteria are outside the scope of this review. The reader is referred to the several reviews on these aspects (27, 28, 225, 227) and the paper by Goren (123*a*).

Fatty Acid Oxidation

The work of earlier investigators gave some clues that the catabolism of fatty acids in mycobacteria is through β-oxidation, but firm evidence in favour of β-oxidation of fatty acids was obtained by Goldman and his co-workers (104, 115) who showed that the cell-free extracts of *M. tuberculosis* H37Ra possess butyryl CoA dehydrogenase, enoyl hydrolase, β-hydroxyacyl dehydrogenase, and β-ketoacyl thiolase. These enzymes have been partially purified; the characteristics of these enzymes are similar to those from the animal tissues. However, the prosthetic group (FAD) of the butyryl dehydrogenase of *M. tuberculosis* H37Ra is easily dissociated, and the apoenzyme does not bind the substrate. Addition of FAD to the apoenzyme restores the enzymatic activity (113).

Biosynthesis of Fatty Acids

Saturated straight-chain fatty acids. It is now known that the steps involved in the biosynthesis of straight-chain saturated fatty acids in mammalian tissues and in bacteria are essentially the same. However, unlike the fatty

acid synthetase from yeast and liver, the fatty acid synthetases from *Escherichia coli* and *Clostridium kluyveri* have been resolved into individual components. One of these, the acyl carrier protein (ACP), has been purified and its role in fatty acid synthesis has been investigated (246). Kusunose et al. (218) demonstrated acetyl coenzyme A (CoA) carboxylase activity in the cell-free extracts of *M. avium* at about the same time that the role of malonate in fatty acid biosynthesis was being resolved by Wakil (392) in pigeon liver. The incorporation of $^{14}CO_2$ into malonate requires acetate (or acetyl phosphate), CoA, and a divalent metal ion; adenosine triphosphate (ATP) inhibits the reaction. The soluble enzyme, which is partially purified, catalyzes the synthesis of fatty acids from acetate-*1*-^{14}C in presence of bicarbonate and ATP (CO_2 has a stimulatory effect), and palmitic acid is the major product. However, the fatty acid synthetase from *M. avium*, unlike that of the avian and mammalian tissues, forms from acetate, fatty acids of longer chain length (up to C_{24}) such as stearic, arachidic, behenic, and lignoceric acids (218, 219). In the presence of malonate, most of the radioactivity from labeled acetate appears in fatty acids higher than palmitic, and lignoceric acid is the major product. Synthesis of myristic and octanoic acids has also been reported in the same organism (77).

Subsequently the demonstration of the synthesis of malonyl CoA from acetyl CoA and of fatty acids from ^{14}C-labeled acetate in *M. tuberculosis* H37Ra, *M. smegmatis*, and BCG established the existence of malonyl pathway of fatty acid synthesis in mycobacteria (284, 287, 407). The fatty acids formed include C_6, C_8, C_{16}, C_{18}, C_{20}, and at least two longer-chain acids in *M. smegmatis* and BCG and still longer-chain acids (C_{16} to C_{32}) in *M. tuberculosis* H37Ra. The fatty acid-synthesizing systems of *M. tuberculosis* H37Ra and *M. smegmatis* 607 require ATP, CoA, Mn^{2+} or Mg^{2+}, and NADPH (or NADPH generating system). The fatty acids synthesized are in the esterified form. Surprisingly, in both the systems, NADH stimulates the incorporation of acetate into fatty acids. Avidin inhibits the synthesis of fatty acids from acetate, and this inhibition could be reversed by biotin. Even high concentrations (1.5 mg/ml) of avidin show only 80% inhibition of the synthesis of fatty acids from acetate in *M. smegmatis* 607, and it was therefore suggested that, in this organism, part of the fatty acid synthesis (about 20%) proceeds by the avidin-insensitive chain elongation process (284). The optimum *p*H for fatty acid synthesis in *M. smegmatis* 607 is 8.6.

The composition of the fatty acids synthesized in *M. tuberculosis* H37Ra shows some interesting features (287). No significant radioactivity from labeled acetate is found in straight-chain, saturated, even-numbered fatty acids having less than 16 carbons or more than 32 carbons, or in unsaturated and branched-chain fatty acids. In particular, neither tuberculostearic acid nor oleic acid, present in the extracts, shows any radioactivity. Hexacosanoic (C_{26}) acid, which is considered to be involved in the synthesis of mycolic acids, is the major product formed.

Recently, two fatty acid synthetases (system I and system II) have been demonstrated in *M. phlei* (48, 251, 252). The first (system I) was purified from the particle-free extracts of *M. phlei* grown in presence of ^3H-β-alanine as a marker for 4'-phosphopantotheine (48). Radioactivity is found in the fatty acid synthetase, indicating that the *M. phlei* synthetase, like the fatty acid synthetases of liver and yeast, has a bound ACP-like component. It is a multienzyme complex and has a high molecular weight of 1.7×10^6. However, the *M. phlei* fatty acid synthetase differs from the fatty acid synthetases described so far in the following properties. (i) It requires a heat-stable stimulating factor (SF) for its activity, and this stimulating factor does not appear to be ACP or acetyl CoA. There is, however, some interdependence between acetyl CoA and SF concentrations required for optimal activity. (ii) Its acyl transferase activity has a much broader specificity. It uses not only acetyl CoA but longer-chain acyl CoA derivatives, such as octanoyl CoA and stearoyl CoA, as primers for malonyl CoA incorporation into long-chain, even-numbered fatty acids (C_{12} to C_{26}). (iii) The fatty acids produced (C_{14} to C_{26}) show a biphasic chain-length distribution, i.e., C_{18} and C_{24} are the major acids produced, and the others are in smaller quantities. (iv) It is unstable in solutions of low ionic strength (phosphate buffer 10 mM or less) and dissociates into smaller units. SF prevents such decay of activity. The ACP of *M. phlei* is present in a bound form (in the fatty acid synthetase multienzyme complex) and also in a free form (251, 252). The bound ACP can be released by alkaline hydrolysis. The ACP (*M. phlei*) has been purified, and its properties have been compared with that of ACP (*E. coli*) (251, 252). The ACP (*M. phlei*) is a low-molecular-weight, heat-stable compound, and its amino acid composition is similar to that of ACP (*E. coli*). The former has no histidine and

has four proline residues compared to only one in ACP (*E. coli*).

In addition to the above synthetase, Bloch and his coworkers (48, 252) observed the presence in *M. phlei* of a second type (system II) of fatty acid synthetase for chain elongation. This resembles the fatty acid synthetases of bacteria in that it is totally dependent on the addition of ACP from either *M. phlei* or *E. coli*. Its molecular weight is less than 250,000. An interesting property of this fatty acid synthetase is that it uses only palmitoyl-CoA or stearoyl-CoA, but not octanoyl-CoA or acetyl-CoA, for chain initiation.

As mentioned previously, the long-chain fatty acid synthesis may also be taking place by an avidin-insensitive chain-elongation process in the crude extracts of *M. smegmatis* 607 (284). The presence of two enzyme systems for the chain elongation of fatty acids in *M. tuberculosis* H37Ra has also been demonstrated (182, 393). In the first one, which is an avidin-insensitive mechanism, acetate-*1*-^{14}C is incorporated into a medium-chain (C_5–C_{10}) acid yielding a fatty acid, two carbons longer than the starting material (C_{n+2} acid from a Cn acid and acetate). In this type of reaction, the stimulation is maximal with octanoate as the substrate. Similar elongation was observed with palmityl CoA also. For this reaction ATP, CoA, a metal ion, and NADH are necessary. ATP is not necessary when fatty acyl CoA and acetyl CoA are used. The elongation appears to occur by the addition of a single C_2 unit to the carboxyl end of the acceptor fatty acid. The second reaction is a direct condensation of two or more molecules of fatty acid (Cn) to give a longer chain (C_{2n} or C_{3n}) acid. For this reaction, a metal ion, ATP, and NADH are necessary. From octanoyl-*1*-^{14}C CoA, radioactivity is found in palmitate and tetracosanoate. Similarly, eicosanoate is the product with decanoyl CoA as the substrate. Matsumara, Brindley, and Bloch (252) reported, in *M. phlei*, a fatty acid synthetase which requires an external supply of ACP. Palmitoyl CoA and stearoyl CoA, when used as primers, show incorporation of malonyl CoA. Octanoyl CoA is found to be less active.

Unsaturated straight-chain fatty acids. Two pathways—aerobic and anaerobic—are known to be involved in the biosynthesis of monounsaturated fatty acids (186, 222, 231, 276, 305). Of these two, the aerobic pathway which brings about dehydrogenation of the corresponding saturated acids in presence of oxygen and NADPH was demonstrated in the particulate fraction of *M. phlei* (94). Unlike the other bacteria having the aerobic pathway, the *M. phlei* system requires Fe^{2+} and a flavine (FAD or FMN), in addition to NADPH and oxygen, for the synthesis of Δ^9-unsaturated fatty acids. NADPH is required in substrate amounts and flavine in catalytic amounts. Stearoyl CoA and palmitoyl CoA are desaturated to Δ^9-octadecenoic and Δ^9-hexadecenoic acids, respectively. The formation of the cis-Δ^9-unsaturated acids in the cell-free system is surprising because, when *M. phlei* cells are grown on palmitic acid-*1*-^{14}C, the product is primarily the Δ^{10}-isomer of hexadecenoic acid (319). The difference between the products obtained with the intact cells and cell-free system in the same organism remains to be explained. The synthesis of unsaturated fatty acids by the particulate fraction is inhibited by sulfhydryl reagents and is irreversibly lost on dialysis or treatment with ammonium sulfate. A recent report (29) that about 5% of the lipids of *M. phlei* contain polyunsaturated fatty acids calls for an evaluation of the biosynthetic process of this group of fatty acids.

Branched-chain fatty acids. The branched-chain fatty acids which occur in mycobacteria may be divided into three groups: (i) acids with one methyl branch in the middle of the chain (tuberculostearic acid group), (ii) acids with three or four methyl branches at the carboxyl end (phthienoic and mycocerosic acid group), and (iii) acids with long branches and one or two oxygenated functions (mycolic acid group).

Methyl branched acids. The biosynthesis of tuberculostearic (10-methyl stearic) acid by *M. phlei* (Fig. 1) proceeds by dehydrogenation of stearic acid to oleic acid and then, by addition of a C_1 unit from methionine (in the form of *S*-adenosylmethionine). Similarly, when palmitic acid is the substrate, the product is methyl

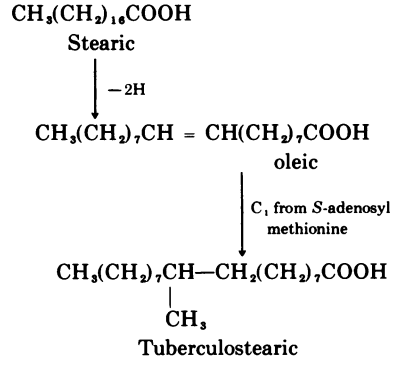


FIG. 1. *Biosynthesis of tuberculostearic acid.*

palmitate (232, 319). The source of the C-methyl group in tuberculostearic acid is shown to be methionine in *M. tuberculosis* H37Ra also (226). However, using whole cells of *M. smegmatis* and cell-free extracts of *M. phlei* and with methyl-labeled methionine, Jaure'quiberry et al. (169, 170) found that in the methyl group of the branched fatty acid only two of the three hydrogens are derived from the methionine methyl group. In support of this observation, 10-methylene stearic acid is identified to be an intermediate in the conversion of oleic acid to tuberculostearic acid. Further, when *M. phlei* cells are incubated with labeled 10-methylene stearic acid, there is radioactivity in tuberculostearic acid. By mass spectrometric studies with deuterated compounds, Lenfant, Audier, and Lederer (229) showed that a shift of H occurs from C10 of oleic acid to C9 during the methylation step. Similar results were reported by Bristol and Schroepfer (49). The conversion of oleic acid to 10-methylene stearic acid and the reduction of the latter to tuberculostearic acid takes place only on the endogenous phospholipid substrates (phosphatidylglycerol, phosphatidylinositol, and phosphatidylethanolamine), and externally added phospholipids do not have any effect (3). However, when partially purified enzyme preparations are used, exogenous phosphatidylethanolamine stimulates the synthesis of alkylated fatty acid derivatives (4). The reduction step requires NADPH but no NADH as the cofactor, and the following reactions are suggested: (i) Oleyl phospholipid + *S*-adenosylmethionine → 10-methylene stearyl phospholipid + *S*-adenosylhomocysteine; (ii) 10-methylene stearyl phospholipid + NADPH + H^+ → 10-methyl-stearic acid + NADP. This enzymatic alkylenation occurs on the fatty acids at position 1 or 2 of the glyceride molecule (5). In addition, a soluble enzyme system from *M. phlei* capable of esterifying fatty acids (carboxyl group alkylation) has also been described (6); oleic acid is the most effective fatty acid acceptor and *S*-adenosyl methionine is the methyl donor.

Campbell and Naworal (58*a*) suggested that leucine and isoleucine can serve as "starters" in the biosynthesis of branched-chain fatty acids, as in other bacteria (186, 222, 231, 276, 305). They also postulated a general scheme for the synthesis of all methyl branched fatty acids involving the participation of a methyl transferase. This scheme, however, needs experimental support.

Multiple methyl branched acids (phthienoic and mycocerosic acids). Yet another pathway seems to be operating for the biosynthesis of mycocerosic acids and phthienoic acids, which are fatty acids with multiple (3 or 4)-branched methyl groups, of which 2,4,6,8-tetra-methyl-octacosanoic acid in a typical example.

In the extracts of *M. smegmatis*, an avidin-sensitive carboxylation of propionyl-CoA to methylmalonyl CoA has been observed, and the propionyl CoA carboxylase therefore may have a role in the biosynthesis of branched-chain fatty acids (341). The whole cells of *M. tuberculosis* H37Ra synthesize 2,4,6,8-tetramethyl octacosanoic acid from a C_{20} straight-chain acid by the successive addition of four molecules of propionic acid (101). The condensation is on the α-position of the propionate molecule (102, 186). In *M. bovis* also, propionate-*1*-^{14}C is incorporated into mycocerosic acids (427).

Phthienoic acids, present only in virulent *M. tuberculosis*, are similar to the mycocerosic acids but have trans α-β unsaturation and are believed to be synthesized by similar mechanisms (102, 186, 226). Multiple-branched (7 to 8 methyl branches) carboxylic acids from mycobacterial sulfolipids have been recently characterized (124), but their biosynthesis is unknown.

Mycolic acids. Biosynthesis of α-smegmamycolic acid (Fig. 2) in the cells of *M. smegmatis* grown in presence of tetracosanoic acid-*1*-^{14}C and ^{14}C-methionine seems to involve incorporation of tetracosanoic acid molecules as such into the carboxyl terminal (24 carbons) and methyl group of methionine into the methyl branch (87). When palmitate-*1*-^{14}C is used in the medium, radioactivity is incorporated into the 60-carbon mycolic acids (211). The mycolic acids of *M. tuberculosis* and *M. bovis* have more branched chains (the branch chain at the α-position derived probably from hexacosanoic acid) and additional oxygen functions such as hydroxyl, methoxy, or carboxy group. The biosynthesis of these acids is not yet fully understood (27, 221, 225). In this connection, it is interesting to recall that the fatty acid synthetases of different species of mycobacteria are capable of synthesizing not only C_{16} or C_{18} acids but also C_{24} and C_{26} acids, which in turn may serve as building blocks for the biosynthesis of more complex mycolic acids. However, the biosynthesis of mycolic acids using cell-free enzymes will throw further light on the details of the mechanism. Phthiocerol, a methoxydiol present in *M. tuberculosis*, is synthesized starting from tetracosanoic acid; methionine serves as methyl donor (100, 186).

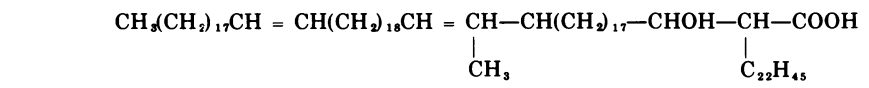


FIG. 2. *Structure of α-smegmamycolic acid.*

Phospholipids

Interest in the phospholipids of mycobacteria, which were originally described by Anderson (13), has in recent years been revived, and their immunological role is being actively studied. The structure of the mycobacterial phosphatides has been recently reviewed (281).

The phosphatides of mycobacteria show a high turnover. In *M. smegmatis* 607 exposed to $^{32}P_i$ for a short time, lysophosphatidylethanolamine and phosphatidylinositol undergo rapid turnover when compared with other phosphatides (343). There is a sudden loss of radioactivity in lysophosphatidylethanolamine, but the radioactivity in phosphatidylinositol increases for 1 hr. In growing *M. phlei* cells, phospholipids have an appreciable turnover (7) but, in contrast to *M. smegmatis* 607, cardiolipin has the highest turnover rate, whereas phosphatidylinositol oligomannoside and phosphatidylethanolamine have low turnover rates. It is possible that these variations are due either to the difference in the species of mycobacteria investigated or technique used. Incorporation of $^{32}P_i$ into the lipids of *M. tuberculosis* H37Ra requires at least 24 hr (165).

The biosynthesis of phosphatidylinositol mannosides in the cell-free extracts of *M. phlei* proceeds in a manner which is not yet completely understood (45, 46, 151). Guanosine diphosphate mannose (GDP-mannose) is the mannose donor and phosphatidylmyoinositol is the acceptor; the major products are three phosphatidylmyoinositoldiamannosides. These are designated as dimannophosphoinositides A, B, and C, which differ from one another in the number of acyl groups contained (4, 3, and 2, respectively). It is assumed that two of the fatty acids are on the glycerol moiety and the rest (in A and B forms) are attached to available hydroxyls of mannose or myoinositol moieties. The enzyme catalyzing the biosynthesis of the dimannosides is present both in the particulate and supernatant fractions, but the former has higher activity than the latter. The enzyme has been solubilized from the particles by treatment with acetone; the solubilized enzyme shows a requirement for external phosphatidylmyoinositol. The enzyme is inhibited by Tween 80; Mg^{2+} stimulates the activity at low concentrations but inhibits at high concentrations. However, the particulate enzyme uses the endogenous phosphatidylmyoinositol itself as an acceptor of GDP-mannose-^{14}C. The particulate fraction of *M. phlei* has another enzyme system which acylates, in presence of ATP, CoA and the fatty acids or fatty acyl CoA, the dimannophosphoinositides (46). The fatty acids incorporated include not only palmitic acid, which is naturally present in the phosphatides, but also myristic, stearic, and oleic acids. The incorporation of tuberculostearic acid is only in traces. However, the recent demonstration that the biosynthesis of tuberculostearic acid from oleic acid occurs in the presence of phospholipids (5) is of interest. In the interconversion of dimannophosphoinositides, the formation of polyacyldimannophosphoinositides (having more than four fatty acids) is also implicated (46). Similar results were obtained by the above authors with BCG. The pentamannophosphoinositide is expected to be formed by further addition of more mannose units to the mannose already attached at position 6 of the myoinositol ring of the dimannophosphoinositide.

In cell-free extracts of *M. tuberculosis* H37Ra, the particulate transmannosylase enzyme system has two independent reactions (356): one for the synthesis of phosphatidylinositomonomannoside (from phosphatidylmyoinositol and GDP-mannose) and the other for the phosphatidylmyoinositoldimannoside (from phosphatidylmyoinositolmonomannoside and GDP-mannose). This conclusion is based on the observation that, when the particulate fraction is incubated with GDP-mannose (i.e., using endogenous acceptor): (i) radioactivity is observed both in the mono- and di-mannosides; and (iii) radioactivity of the dimannoside is in the mannose moiety attached to the 6-position of the myoinositol. In the above reaction, the major product is a phosphorylated isoprenylalcohol, the structure of which was proposed as mannosyl-1-phosphoryl decaprenol (357).

Two phospholipases—phospholipase A and lysophospholipase—are present in the membrane fraction of *M. phlei* (277). Phosphatidylethanolamine, phosphatidylcholine, and cardiolipin serve as substrates, but the former is hydrolyzed more rapidly. The *p*H optima are 5.1 to 5.3 and 4.0 to 4.2, respectively. According to Ono and Nojima (278), phospholipase A is also a regulatory enzyme with Fe^{3+} as a negative effector. The purified phospholi-

pase A is inhibited by ferric ion (0.12 mM Ki). The substrate (phosphatidylethanolamine) saturation curve shows a sigmoid shape. The enzyme undergoes reversible interconversions. In presence of Fe^{3+}, it exists in an inactive precipitate form (aggregate) but assumes an active soluble form on the addition of ascorbic acid.

The recent demonstration of the presence of phosphonolipids and glyceryl ethers in mycobacteria and the observation that the amount of these constituents varies in different species of mycobacteria make the study of their metabolism interesting (62, 296). There is some evidence that *M. phlei* can slowly utilize 2,3-dihydroxypropylphosphonate for growth (253).

Cord Factor

Cell-free extracts of *M. tuberculosis* H37Ra synthesize trehalose-6-phosphate (a precursor for cord factor) from glucose-6-phosphate and uridine diphosphate glucose (116). Mycoribnin, an oligoribonucleotide, containing adenine and guanine is a noncompetitive inhibitor of this transglycosylase reaction.

Mycosides A and B

The biosynthesis of the aglycon (a phenol glycol) of the mycosides A and B has been investigated by Gastambide-Odier, Sarda, and Lederer (103). The phenolic ring of the phenol glycol comes from tyrosine and the methyl branches from propionic acid.

Lipopolysaccharide

The cell-free extracts of *M. phlei* catalyze the transfer of methyl groups from *S*-adenosylmethionine methyl-^{14}C to endogenous acceptor yielding a labeled lipopolysaccharide (88). This polysaccharide methyl transferase exhibits a *p*H optimum of 7.0 and an apparent K_m of 0.1 mM for *S*-adenosylmethionine when amylooligosaccharides are used as the acceptors.

Effect of isoniazid on lipid metabolism. The mode of action of antitubercular drugs has been discussed in another section of this review. However, the effect of isoniazid on lipid metabolism is pertinent. The elegant work of Brennan, Rooney, and Winder (47) shows that the methods used for extraction of lipids and their fractionation influence the results obtained. Isoniazid produces three distinct effects on the lipid metabolism of BCG. (i) It reduces the incorporation of radioactivity from ^{14}C-glycerol into ether-soluble bound lipids like mycolic acids and phospholipids, (ii) it decreases the amount of longer-chain acids (above C_{16}) and increases the amount of shorter chain acids, and (iii) it diminishes the amount of triglyceride extracted with ethanol-ether in the classical Anderson-type procedure, leaving an increased amount of the chloroform-soluble and bound lipids. This redistribution effect is masked when a more vigorous lipid extraction procedure of Brennan and Ballou (45) is followed. This was, therefore, considered to be due to alteration in the properties of the cell wall as a result of inhibition of mycolic acid synthesis by isoniazid (408). Singhvi and Subrahmanyam (327) also observed that both isoniazid and dihydrostreptomycin inhibit the incorporation of $^{32}P_i$ into all the major phospholipids of *M. avium*.

Other Lipids

Although cholesterol could not be detected in mycobacteria, the organisms have the ability to decompose cholesterol (157, 158, 200). *M. smegmatis* is capable of esterifying some hydroxy sterols with fatty acids and succinic acid (320, 321).

ELECTRON TRANSPORT AND OXIDATIVE PHOSPHORYLATION

Studies on electron transport and oxidative phosphorylation have been carried out extensively with *M. phlei* by Brodie and his colleagues (51, 52, 56), but not much is known about other species of mycobacteria.

Preparation, Properties, and Composition of the Particulate (Membrane) Fraction

Electron transport activity in *M. phlei* is localized in the membrane or particulate fraction (52, 55, 397, 398). Lysozyme treatment, which has yielded from *Micrococcus lysodeikticus* (164) a single fraction exhibiting both oxidation and phosphorylation, has not been quite successful in lysing mycobacteria unless lysozyme is included in the growth medium for several hours before harvesting the cells (1, 257, 367, 403). Relatively more harsh methods such as sonic treatment (see Table 1) employed in the case of other microorganisms (154, 185, 288) have been used for mycobacteria. Consequently in *M. phlei*, as in many other bacteria, both particulate and soluble supernatant fractions are necessary for electron transport and oxidative phosphorylation. Recently in the laboratory of one of us, it has been possible to obtain a particulate fraction from *M. smegmatis* which oxidizes malate (by the nicotinamide nucleotide pathway) and shows coupled phosphorylation (296). The method consists in treating the cells with lysozyme, subjecting them to osmotic shock, and

TABLE 1. *Methods used in the preparation of cell-free extracts*

Organism	Methods in disintegration	Activities demonstrated[a]	Reference no.
Mycobacterium phlei ...	Sonic oscillation	Enzymes of the electron transport chain, oxidative phosphorylation and tricarboxylic acid cycle; fatty acid synthetase	48, 54, 149, 397
M. smegmatis	Grinding with glass powder, sonic oscillation and treatment with lysozyme	NADH-diaphorase, enzymes of the tricarboxylic acid cycle and glycolytic pathway; NADH- and cytochromic-oxidases; malate oxidation; phosphorylation	61, 257, 326
M. avium	Grinding with sea sand	Enzymes of fatty acid synthesis	219
M. tuberculosis H37Ra ..	Grinding with alumina, colloid mill and sonic oscillation	NADH- and NADPH-oxidases, enzymes of glycolysis and the tricarboxylic acid cycle	114, 161, 420
M. tuberculosis H37Rv ..	Sonic oscillation	Enzymes of the tricarboxylic acid cycle, glycolytic, glyoxylate and hexose monophosphate pathways, NADH- and NADPH-oxidases, NADase and NADH-diaphorase	121, 162, 272, 350
M. bovis BCG	Grinding with quartz sand or alumina, and high pressure disruption	Fatty acid synthetase and acetyl CoA carboxylase; isolation of ribosome and their proteosynthetic ability	217, 380, 408

[a] Abbreviations: NADH, nicotinamide adenine dinucleotide (reduced form); NADPH, nicotinamide adenine dinucleotide phosphate (reduced form); NADase, nicotinamide adenine dinuclease; CoA, coenzyme A.

sonically treating them for a very brief period. The membranes isolated from *M. smegmatis* by Cesari et al. (61) and Mison et al. (257) contain NADH oxidase or diaphorase activity, but nothing is known about their phosphorylation.

The *M. phlei* particles—70 to 180 nm long—swell in hypotonic solutions and tend to clump in hypertonic solutions (55). They contain some dehydrogenase (most of them are in the supernatant fraction), NADH (exogenous NADH) oxidase, NAD, vitamin K_9H, cytochromes *b*, *c*, *a*, a_3, and *o*, nonheme iron, and lipids (22, 55, 76, 96, 216, 308, 398, 399). The presence of phospholipids also has been reported in *M. tuberculosis* H37Ra (114). The cytochrome composition of different species of mycobacteria reveals some interesting features. Cytochromes *b*, *c*, and *a* are common to all the species of mycobacteria grown in vitro, namely *M. smegmatis*, *M. phlei*, BCG, *M. avium*, *M. tuberculosis* H37Ra, and *M. paratuberculosis* (52, 188, 217). In addition to these cytochromes, *M. smegmatis* and *M. phlei* contain a carbon monoxide-binding pigment similar to cytochrome *o* (217, 308). However, cytochromes could not be detected in BCG grown in vivo and in *M. leprasmurium* isolated from leprous nodules (217).

Respiratory Chain

Using techniques similar to those adopted with mitochondria, Asano and Brodie (22) determined the sequence of the components of the respiratory chain. The respiratory chain shown in Fig. 3 is based on the scheme given by Brodie and Adelson (52) incorporating the results of subsequent studies of Brodie and his co-workers (212, 216, 279, 308). Both oxidation and coupled phosphorylation, with malate and other NAD-linked substrates, require the particulate as well as the supernatant fractions (21, 22). With succinate as the substrate, particles alone show some oxidation and associated phosphorylation; addition of the supernatant fraction, however, stimulates both oxidation and phosphorylation (21, 50). In the reconstituted system containing both the particulate and supernatant fractions, cytochromes *b*, *c*, *a*, + a_3, and *o* are reduced with succinate, malate, and β-hydroxybutyrate as the substrates. Thus, both the succinate and NAD-linked pathways converge at cytochrome *b* level, and then the electrons flow cytochromes *b* to *c* to *a* + a_3 and to oxygen. The exact location of cy-

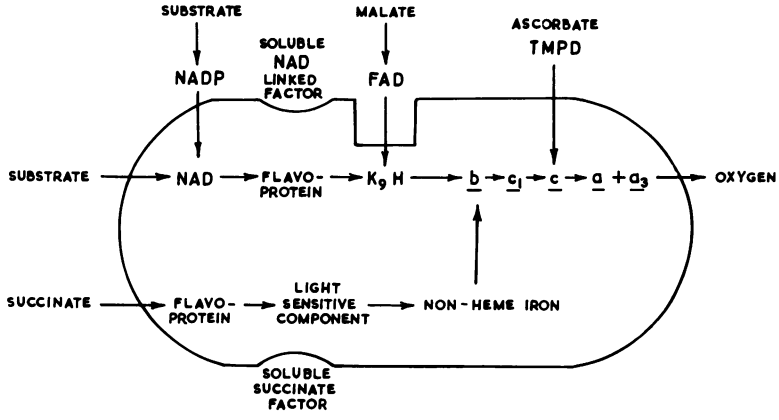


FIG. 3. *Respiratory chain in Mycobacterium phlei. K_9H, Naphthoquinone vitamin K_9H; b, c_1, c, a, and a_3, the cytochromes; TMPD, tetramethylparaphenylenediamine.*

tochrome *o* in the chain is not yet determined (308). Before cytochrome *b*, the NAD and the succinate segments show some differences in addition to specific flavines.

The naphthoquinone, vitamin K_9H, is considered to play an important role in the NAD-linked pathway and the malate-vitamin K reductase pathway (shown in Fig. 3 as malate FAD pathway) for the following reasons (51, 52, 56, 135). (i) Both oxidation and phosphorylation are lost with all the substrates on exposure of the particulate and supernatant fractions to near ultraviolet light (360 nm), which is believed to destroy the natural naphthoquinone but does not apparently affect the other cofactors or the structural integrity of the particulate fraction. (ii) If the natural naphthoquinone or vitamin K_1 or some of its homologues suspended in phospholipid are added to such an irradiated system, both oxidation and phosphorylation are restored with NAD-linked substrates and malate-vitamin K reductase pathway but not succinate. (iii) Under phosphorylating conditions, incorporation of tritium from the medium occurs only into vitamin K_1, but not into 2,3 dihydrophytyl vitamin K_1 or lapachol. In view of the fact that FAD is required for quinone reduction in irradiated particles, vitamin K appears to be located between the flavine and cytochrome *b*. However, studies by Di Mari et al. (72) and Snyder and Rapoport (337) have clearly indicated that, in the irradiated *M. phlei* system, tritium incorporation into vitamin K_1 is 0.1% or less.

The succinate segment is more sensitive to light (360 nm) than is the NAD segment. Neither naphthoquinones nor benzoquinones restore succinoxidase activity lost by irradiation (53), but addition of fresh supernatant fluid or extract of boiled whole cells restores 50 to 60% of the original activity lost by irradiation (212). The protein-bound succinate factor is light-sensitive but, after partial purification, it is thermostable and resistant to irradiation. It acts at a site between flavine and cytochrome (212). A similar factor is present in rat liver mitochondria (212, 213). The cyanide-sensitive metal in the succinate chain has been shown to be nonheme iron; it is located between the light-sensitive component and cytochrome *b* (215, 216). Krishna Murti et al. (213) have succeeded in dissociating succinate dehydrogenase (SDH) from the particles by alkali extraction and recombining the fresh SDH (rich in labile sulfur and nonheme iron) with alkali-treated particles.

In addition to the NAD and succinate pathways, a third pathway has been shown to occur in *M. phlei*, which accounts for malate oxidation requiring naphthoquinone and FAD but not NAD and entering the main respiratory chain at the naphthoquinone level (22). The enzyme responsible for this reaction, malate-vitamin K reductase, is present both in the particulate and in the supernatant fractions. The latter has been used as a convenient soluble system for studying the mechanism of phosphorylation in *M. phlei* (26, 347, 349), unlike the NAD-independent malate dehydrogenases reported in other sources (66, 92, 235, 370). In 2n-nonyl hydroxyquinoline *N*-oxide (NHQNO)-blocked particles, with ascorbate in presence of tetramethyl paraphenylene-diamine (TMPD) as the substrate, the electrons enter at cytochrome *c* level and then flow to cytochrome $(a + a_3)$. However, with succinate as the substrate, the electrons bypass cytochrome *b* and reenter the respiratory chain at cytochrome *c* level (212, 279). The role of elec-

tron-transport component with NADP-like activity isolated from *M. phlei* by Sutton (352) needs further study.

Phosphorylation

As in the case of other bacteria, the P/O values obtained in *M. phlei* are lower than those reported with mitochondria. In the reconstituted system wherein particulate fraction and ammonium sulfate fractionated supernatant fluid are used, the P/O values with the malate-vitamin K reductase and succinate chains are between 0.4 and 0.8, whereas, with the NAD-linked substrates β-hydroxybutyrate and ethanol, the values are 1.11 and 1.23, respectively. The low P/O values are attributed to loss of sites of phosphorylation and the presence of nonphosphorylating electron-transport bypass reactions (23). The bypass mechanisms appear to play a role in the oxidation of exogenous NADH or NADPH with which the electrons can be transferred directly to oxygen (entirely bypassing the respiratory chain) or reenter the chain at a higher oxidation reduction level, depending on the electron acceptor used (23, 24, 38, 39, 54, 56, 348). The phosphorylation is sensitive to uncoupling agents like dinitrophenol, *m*-chlorocarbonyl cyanide phenylhydrazone, pentachlorophenol, dicumarol, and atebrin but not to oligomycin (23).

Sites of phosphorylation. Brodie and coworkers (reviewed by Brodie and Adelson, 52) demonstrated three sites of phosphorylation associated with the respiratory chain in *M. phlei*. In cell-free systems of *M. phlei*, the P/O values for the oxidation of succinate or malate (using FAD-mediated malate-vitamin K reductase) are similar and range from 0.4 to 0.8, whereas the same system gives P/O values greater than 1.0 when NAD-linked substrates like β-hydroxybutyrate or ethanol are oxidized. This indicates the presence of one phosphorylation site on the respiratory chain between NAD and the point of interaction of succinate or malate (FAD)-reduced vitamin K, and another on the oxygen side of this point. Further proof of the first site of phosphorylation comes from the observation that the phosphorylation associated with the oxidation of an NAD-linked substrate like β-hydroxybutyrate in presence of lapachol is sensitive to amytal, whereas the oxidation of exogenous NADH in presence of lapachol is insensitive to amytal. By using the *M. phlei* system irradiated with UV light (360 nm), which destroys both oxidation and phosphorylation, and studying the restoration of these two activities with vitamin K_1 and some of its analogues, it has been shown that the second site of phosphorylation is in the quinone-cytochrome *b* region of the chain. In presence of NHQNO, which blocks the respiratory chain at cytochrome *b*, the oxidation of ascorbate in presence of TMPD (which enters the chain at cytochrome *c* level) is accompanied by phosphorylation sensitive to dinitrophenol. This indicates the presence of the third site of phosphorylation between cytochrome *c* and oxygen. Thus, in the case of *M. phlei*, the number and location of the coupling sites appear to agree generally with those of mammalian mitochondria (23, 52).

Coupling factors. A factor which shows phosphorylation when added to purified nonphosphorylating malate-vitamin K reductase and which stimulates phosphorylation coupled to malate oxidation when added to particles washed in the absence of magnesium has been purified from the supernatant fraction of *M. phlei* (347, 349). Subsequently, four factors called bacterial coupling factors (BCF_1 to BCF_4) were isolated from *M. phlei* (40, 149, 150). Two of them (BCF_1 and BCF_4) are from the particulate fraction. BCF_4 separates as a white turbid layer when the particles are subjected to sucrose density gradient centrifugation in the absence of Mg^{2+} (149). When the bottom or "heavy" layer (containing most of the NADH oxidase activity and cytochromes) and BCF_4 are recombined in presence of Mg^{2+} and soluble coupling factors, phosphorylation coupled to NADH oxidation occurs. BCF_4 is essential for phosphorylation with all the substrates and therefore common to all the sites of phosphorylation (149). BCF_1 is released from the particles by urea treatment (150) and appears to participate in reactions common to both succinate and NAD-linked pathways. This, however, is not effective with exogenous NADH or ascorbate-TMPD and is different from the soluble coupling factors (150). Bogin et al. (40) purified (from the supernatant fraction) BCF_2 and BCF_3 which operate at different phosphorylating sites. The coupling factors, BCF_1, BCF_2, and BCF_3, seem to be site-specific because they are required in addition to BCF_4 for different sites (40, 149, 150). The purified coupling factors, BCF_1, BCF_2, and BCF_3, are proteins and contain some adenosine triphosphates, adenylate kinase, ^{14}C-ADP-ATP-, and ^{32}Pi-ATP exchange activities. They have no significant effect on the level of oxidation. The coupling factors from *M. phlei* and beef heart mitochondria are interchangeable (42), and there is interaction between the electron transport particles of *M. phlei* and of mammalian systems after cytochrome *b* and before cyto-

chrome *c* (39). Treatment of particles with either heat or trypsin or a combination of both stimulates phosphorylation (41, 43). The significance of these treatments vis a vis the role of coupling factors in phosphorylation is not yet fully known.

Intermediates of oxidative phosphorylation at the naphthoquinone level of the respiratory chain. The mechanisms involved in the formation of ATP from reduced naphthoquinone have been reviewed (51). Watanabe and Brodie (394) reported the formation of a quinol phosphate similar to, but not identical with, the 6-chromanyl derivative of vitamin K_1 (25, 199, 315). Since there is no significant incorporation of tritium from the medium into the quinone, the quinone methide mechanisms may not be operating, although chromanol formation could still be possible (72, 337).

Electron Transport in Other Mycobacteria

Sonic extracts (10,000 × *g* supernatant) of *M. tuberculosis* H37Rv have NADH oxidase and NADPH oxidase activities, NADH and NADPH cytochrome *c* reductases and NADH diaphorase and NADPH diaphorases (350). These extracts, however, oxidize citrate, isocitrate, and *cis*-aconitate only in the presence of an artificial electron acceptor like phenazine methosulfate or menadione. This may mean that some component(s) of the electron transport chain might have been destroyed during the sonic treatment (350). It is also possible that part of the respiratory chain might have been bypassed in the electron transport associated with these substrates.

In *M. tuberculosis* H37Ra, NADH oxidase and NADPH oxidase activities have been demonstrated in the sonic extracts (161, 272). Goldman and his co-workers have studied in greater detail the NADH oxidase activity of the particulate fraction of *M. tuberculosis* H37Ra (118, 188, 273, 325, 418). NADH oxidase activity is not coupled to phosphorylation. On addition of NADH to the NADH oxidase under anaerobic conditions, cytochromes *c* and $a + a_3$, but not cytochrome *b*, of the particulate fraction are reduced. The NADH oxidase activity is inhibited by cyanide, NHQNO, PCMB, *N*-ethylmaleimide and dicumarol. This indicates that, under the experimental conditions of Kearney and Goldman (188), at least part of the electron transport chain around cytochrome *b* seems to have been bypassed.

Heinen et al. (145) have compared the properties of NADH diaphorases of *M. tuberculosis* H37Ra solubilized from the particulate fraction by various procedures, such as ultrasonic treatment, freezing and thawing, digitonin or alkali extraction, with those in the soluble supernatant fraction. The two types of diaphorase have the same electron acceptor specificity, but they differ from each other in their physical properties, such as response to heat inactivation, etc. The authors have therefore concluded that the soluble NADH diaphorase is derived from the comminution of the NADH oxidase of the cytoplasmic membrane. The variations between the different solubilized diaphorases are attributed to differences in the size of the fragments.

In *M. smegmatis*, NADH oxidase and cytochrome *c* oxidase activities have been demonstrated in a membrane fraction sedimenting between 5,000 and 25,000 × *g* (257), and diaphorase activity has been demonstrated in the membrane mesosome fraction (61).

Regulation of Electron Transport and Coupled Phosphorylation

The particulate NADH oxidase of *M. tuberculosis* H37Ra is an allosteric enzyme (418, 419, 420). NADH oxidase is activated specifically by adenosine monophosphate (AMP) (half maximal at 0.2 mM) but not by adenosine diphosphate (ADP), ATP, 3′5′-cyclic AMP or cytidine monophosphate; deoxyadenosine monophosphate is only 30% as effective as AMP; AMP protects NADH oxidase from alkali (*p*H 8.5) inactivation. According to Worcel, Goldman, and Cleland (420) AMP brings about a conformational change of protein structure, because the enzyme remains in the active state even after the AMP is dissociated from the enzyme. NAD catalyzes the reversion of the AMP-activated enzyme to the native state. Based on the kinetic data, it was suggested that there is one site for AMP but two sites for NADH—one allosteric and another catalytic on the native protein. Neither inorganic phosphate nor Mg^{2+} is required for the stimulation of NADH oxidase by AMP.

The phosphate acceptor system has a regulatory role in *M. phlei* when Sephadex (G-25)-treated particles (called depleted particles) are used (307). Inorganic phosphate (P_i) and AMP stimulate (the former more than the latter) the oxygen uptake with NADH (generated with alcohol dehydrogenase system). ADP, which inhibits the oxidation in the absence of P_i, stimulates the oxygen uptake in presence of P_i, and ATP shows inhibition of the latter. The stimulation is on the reduction of cytochromes $a + a_3$ but not of cytochromes *b* and *c*.

The oxidation of exogenous NADH in *M. phlei* and its regulation have also been studied

(38, 39). As in the case of *M. tuberculosis* H37Ra (420), the particulate NADH oxidase (both cyanide-sensitive and cyanide-insensitive) are inhibited by NAD (allosteric), and this inhibition is relieved by AMP. However, the *M. phlei* NADH-oxidases show some differences. Both the cyanide-sensitive and the cyanide-insensitive particulate NADH oxidases are stimulated by P_i and fumarate. Furthermore, the NAD inhibition also is reversed by P_i or fumarate; P_i, fumarate, and AMP do not stimulate the soluble NADH oxidase whereas NAD shows some inhibition. Since NADH can be oxidized by many pathways, Bogin et al. (38, 39) suggest that the levels of NADH and NAD, as well as the level of AMP and P_i, may play an important role in the regulation of the pathway utilized for NADH oxidation. Thus the basic mechanism is similar to that suggested by Worcel et al. (420), although the role of inorganic phosphate in *M. tuberculosis* H37Ra in this context needs a closer study.

OTHER ASPECTS OF CARBON METABOLISM

Aromatic Compounds

Mycobacteria can utilize a number of aromatic compounds. Gale (95) showed as early as 1952 that mycobacteria oxidize benzoate directly to catechol without the intermediate formation of salicylate. Whereas *M. fortuitum* is capable of converting salicylate to catechol (385), *M. smegmatis* cannot use salicylate for growth or metabolize it to any great extent (303). When *M. smegmatis* is grown on shikimic acid as sole source of carbon, salicylic acid, anthranilic acid, and 3,4-dihydroxybenzoic acid are excreted into the medium. Since there is no evidence that aromatic compounds such as anthranilic acid or tryptophan give rise to salicylic acid, it was concluded that in this organism salicylic acid arises by the shikimic acid pathway via chorismic acid (303). The salicylate accumulated is postulated to condense with serine to form *N*-salicyloylserine, followed by ring closure to form the 2-(*O*-hydroxyphenyl)-2 oxazoline-4-carboxylic acid moiety of mycobactin. Under conditions of iron deficiency there is an increase in the amount of salicylic acid excreted—an observation which cannot be explained at the present moment.

Guerin et al. (133) have studied the metabolism of benzoic, phenylacetic, and phenylbutyric acids by whole cells and cell-free extracts of *M. phlei*. After incubation of ^{14}C-labeled acids with the cell-free extracts, a strongly radioactive neutral fraction consisting of fatty acid esters of the corresponding aromatic alcohols has been isolated.

Hydrocarbon Utilization

Hydrocarbon utilization seems to be an intrinsic capability of mycobacteria, but practically nothing is known about the mechanism by which hydrocarbons are utilized by them. An attempt to understand this has been made by Lukins and Foster (240) who found that *M. smegmatis* 422 produces the homologous methyl ketones during the oxidation of propane, *n*-butane, *n*-pentane, or *n*-hexane. Aliphatic alkane-utilizing mycobacteria are able to grow at the expense of several aliphatic methyl ketones as sole sources of carbon. An oxygenase reaction is postulated for the attack on methyl ketones.

Oxidation of Phenolic Compounds

The oxidation of phenolic compounds by *M. leprae* has been studied by Prabhakaran and co-workers (290, 295). *M. leprae* isolated from infected human tissues oxidizes D- and L-3,4-dihydroxyphenylalanine, a property not possessed by other mycobacteria (290, 291, 294). This oxidation occurs only under aerobic conditions, and the intermediate product is indole-5,6-quinone. In the range of substrates utilized and in the nature of the products formed, the phenoloxidase in *M. leprae* appears to be different from that obtained from mammalian and plant sources. Compounds which bind copper, for instance, diethyl dithiocarbamate, inhibit the enzyme activity markedly (293, 295). Therefore, it has been suggested that nontoxic inhibitors of phenol oxidase in leprosy bacilli may be of value in developing a rational approach to the chemotherapy of the disease, especially since dihydroxyphenylalanine is present in tissues of the human body (like skin and nerve) where the organisms proliferate.

Beaman and Barkodale (33) have isolated, in pure culture, anaerobic corynebacteria (propionibacteria) from the plasma of tuberculoid leprosy and from lepromata of cases of lepromatous leprosy and found that these organisms, like the *M. leprae* shown earlier, exhibit phenoloxidase activity. The enzyme was also inhibited strongly by diethyl dithiocarbamate.

NITROGEN METABOLISM

Nitrate Metabolism

De Turk and Bernheim (71) were the first to

report that nitrate as well as nitrite and hydroxylamine are assimilated by washed suspensions of BCG. In an extensive study of nitrate reduction by mycobacteria, Virtanen (390) has reported that all of the species investigated reduce nitrate to nitrite, though quantitative differences are observed; there was no reduction of nitrite when cell suspensions were examined in buffer solutions. Nitrate nitrogen is readily utilized by *M. butyricum*, *M. smegmatis*, and *M. tuberculosis* H37Ra, while both nitrite and nitrate are utilized as sole sources of nitrogen by *M. tuberculosis* H37Rv (144). Nitrate reductase of *M. tuberculosis* is inhibited by tungstate, but this inhibition is reversed by molybdate. It is a particulate enzyme present constitutively in the organism (32). The enzyme is specific for NADH, and NADPH cannot replace NADH; 2,heptyl-4,hydroxyquinoline-*N*-oxide, a strong inhibitor of the oxidation of cytochrome b_1 inhibits the enzyme by 70% at a concentration of 1 μm. Reduction does not go far beyond nitrite, which is toxic to the cell, and therefore the authors have concluded that "its physiological properties fit the pattern of the so-called dissimilatory reduction, a process in which nitrate only interferes in the normal energy-yielding reactions of the cell by acting as a non-essential electron acceptor." If this is true, it is necessary to look for other enzyme systems which metabolize nitrate, since it is used as a sole source of nitrogen for growing mycobacteria.

Ellfolk and Katunuma (84) have detected the presence of an ammonia-activating enzyme in *M. avium:* ATP + $NH_3 \rightleftharpoons$ AMP ~ NH_2 + pyrophosphate.

Amino Acids and Amides

General. Our knowledge of amino acid metabolism in mycobacteria has not changed in principle in the period covered by this review, though many details of the metabolism have, of course, been studied. When ^{14}C-labeled acetate is incorporated into the growth medium, the highest specific activities are found in glutamic acid, aspartic acid, proline, isoleucine, and arginine (377). The distribution of activity in different amino acids is dependent on the position of the labeled atom in acetate.

The utilization of amino acids during growth of *M. tuberculosis* H37Ra in rotary cultures has been studied by Lyon et al. (243). The efficiency of amino acids in promoting growth is in the order: alanine $\gg$ glutamate $>$ asparagine $>$ aspartate. Utilization of asparagine during early growth is greater than of alanine and glutamate, and during asparagine metabolism extracellular amino acids are accumulated in the medium. The authors suggested that during the metabolism of asparagine by the H37Ra strain in rotary cultures, metabolic controls may be exerted which impede protein synthesis. In contrast to *M. tuberculosis* H37Ra, *M. smegmatis* 607 grows well with all the nitrogen sources. A comparison with the H37Rv strain of *M. tuberculosis* is also desirable.

The uptake of L-glutamate shows Michaelis-Menten kinetics in *M. avium* whereas that of D-glutamate in both *M. avium* and *M. smegmatis* is accomplished by a passive process showing diffusion kinetics (422, 423). The uptake of D-alanine in *M. smegmatis* is an active process displaying saturation kinetics characteristic of enzyme function, while the uptake of L-alanine, L-glutamine, and D- and L-valine takes place by both the active and passive processes described above. The passive process is, however, sensitive to sulfhydryl blocking agents and shows competition among structurally related amino acids, suggesting that the passive process is a facilitated diffusion.

The cell-free extracts of *M. tuberculosis* H37Rv and BCG catalyze the transfer of amino groups from L-asparagine, DL-aspartic acid, L-isoleucine, L-valine, L-leucine, L-phenylalanine, L-tryptophan, DL-norleucine, and DL-norvaline to α-ketoglutarate (318). *M. avium* also possesses similar transaminase activity but cannot utilize L-phenylalanine and L-tryptophan as substrates.

Asparagine and aspartic acid. Asparagine is the preferred source of nitrogen for the growth of mycobacteria. From the uptake and distribution of labeled carbon from ^{14}C-asparagine by *M. tuberculosis* H37Ra it was shown that asparagine supplies not only nitrogen but also carbon to growing cells (245). The carbon of asparagine is rapidly taken up and extensively utilized by this organism. Washed cells as well as cell-free preparations of *M. tuberculosis* H37Rv, H37Ra, and BCG deamidate asparagine to aspartic acid and ammonia (137, 197). Ott (280) has described asparaginase from *M. tuberculosis* H37Ra and *M. smegmatis* and has determined the kinetics for the formation of L-aspartic acid both with whole cells and cell-free preparations. A single peak of maximal enzyme activity is found at *p*H 8.5, and the two strains of mycobacteria show only quantitative differences in asparaginase activity. In contrast, Jayaram, Ramakrishnan, and Vaidyanathan (172) have demonstrated two L-asparaginases with *p*H optima 9.0 and

9.6, respectively, in *M. tuberculosis* H37Ra and a single enzyme exhibiting maximal activity at *p*H 9.0 in *M. tuberculosis* H37Rv. The two enzymes (*p*H 9.0 and 9.6 enzymes) differ also in kinetic properties, inhibition by L-aspartic acid, and sensitivity to general enzyme inhibitors. The *p*H 9.6 enzyme, characteristic of the H37Ra strain is inhibitory to the growth of Yoshida ascites tumor in rats (342). This enzyme is inducible in the presence of L-asparagine in the growth media (171). Besides, an aspartotransferase, which specifically catalyzes the transfer of the amido group of L-asparagine to hydroxylamine, forming aspartohydroxamic acid, is shown to be present in *M. tuberculosis* H37Ra but absent from H37Rv (173). The enzyme has been separated and freed of L-asparaginase and glutamotransferase; the purified enzyme exhibits maximum activity at *p*H 9.0. The observations on asparaginase and aspartotransferase represent the first examples of qualitative enzymatic differences between the virulent and avirulent strains of *M. tuberculosis*, but what relevance these enzymes have to the virulence of the organism is not known. In addition to the specific aspartotransferase, another nonspecific transferase which acts on both asparagine and glutamine is present in both strains. The enzyme has a *p*H optimum of 9.6.

The presence of aspartokinase (which catalyzes the conversion of L-aspartic acid to β-aspartylphosphate) and homoserine dehydrogenase (which catalyzes the reduction of L-aspartic acid β-semialdehyde to homoserine) in *M. album* has been reported (184). The synthesis of aspartokinase is repressed by DL-threonine and L-methionine, and the activity is inhibited by DL-threonine and DL-allothreonine. The synthesis of homoserine dehydrogenase is repressed by DL-threonine and DL-valine, and its activity is inhibited by DL-threonine, DL-isoleucine, L-valine, L-methionine, or L-phenylalanine. The activity of this enzyme is stimulated by Na^+ and K^+ in the reverse reaction and inhibited slightly in the forward reaction.

Glutamine and glutamic acid. Glutamine can substitute for asparagine as nitrogen source for the growth of mycobacteria, though the yield is slightly lower. The extracts of *M. phlei* possess glutamotransferase activity and hydrolyze aspartohydroxamic acid, but do not have aspartotransferase and glutamo- and asparto-synthetases. The transfer and hydrolytic reactions, therefore, may be catalyzed by different enzymes (130). *M. tuberculosis* also possesses aspartotransferase and glutamotransferase activities which are distinct from each other, but not aspartosynthetase (172). Washed cell preparations of *M. tuberculosis* H37Ra and *M. smegmatis* 607 grown in Sauton's medium oxidize glutamate after a lag period, but when the cells are grown in a modified medium containing glutamate there is no lag (244). The lag is due to the induction of a glutamate transport system.

Prabhakaran and Braganca (292) have reported that *M. leprae* separated from human lepromatous nodules possesses glutamic acid decarboxylase activity, and the product of the enzyme reaction, γ-aminobutyric acid occurs in skin lesions of leprosy as a product of the bacillary metabolism. They could detect the enzyme in *M. phlei* and *M. kedrowsky* but not in *M. smegmatis* and *M. tuberculosis* H37Rv.

Alanine. Lyon and Hall (242) have recently reported that L-alanine can substitute for L-asparagine as nitrogen source for growing *M. tuberculosis*, though the growth rate with the former is lower. The purification and properties of dehydrogenase from *M. tuberculosis* H37Ra and H37Rv have been described (112, 299). The enzyme is specific for L-alanine and requires NAD for activity. The enzyme is also present in *M. phlei*, *M. smegmatis*, and *M. lacticola;* the properties of the enzymes from the two strains of *M. tuberculosis* and the saprophytic strains are similar (302).

Lysine. The production of lysine from diaminopimelic acid by cell-free extracts of *M. tuberculosis* H37Rv and H37Ra strains has been demonstrated by Willett (402). Diaminopimelic acid is decarboxylated to give lysine, and there is no difference in the activities of the virulent and avirulent strains.

Isoleucine and L-valine. In addition to the known enzymes of the isoleucine-valine biosynthetic pathway, cell-free extracts of *M. tuberculosis* possess a new enzyme, acetohydroxy acid isomerase which isomerizes α-acetolacetate to α-keto β-dihydroxyisovalerate, which is, in turn reduced to α-β-dihydroxyisovalerate in the presence of NADPH (9). This is an alternate pathway to convert α-acetolacetate to α-β-dihydroxyisovalerate, mediated by an isomerase, followed by a reductase, in addition to the usual isomeroreductase pathway. The isomerase has been purified about 100-fold and separated from reductase and isomeroreductase (10). The enzyme requires specifically L-ascorbic acid and sulfate for its activity. Since the requirement of this enzyme for L-ascorbic acid is specific, it should be possible to use the enzyme in a test system for L-ascorbic acid and its antagonists. The isomeroreductase from *M. tuberculosis* H37Rv

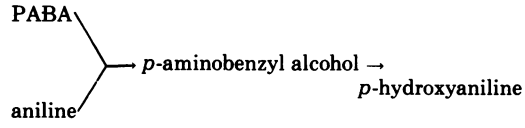
has also been purified about 40-fold (11). L-Ascorbic acid has no effect on the purified isomeroreductase, unlike the isomerase. Thus, the three enzyme activities reside in three separate proteins. Since ascorbic acid is present in extracts of *M. tuberculosis* (12), this vitamin may be a natural cofactor in the isomerization reaction.

L-Valine inhibits acetohydroxy acid synthetase, the first common step in the biosynthesis of isoleucine and valine and, when added to the growth medium, inhibits the growth of *M. tuberculosis* H37Rv (9). On the other hand, in *M. pellegrino* valine coordinately increases the levels of acetohydroxy acid synthetase, dihydroxy acid dehydratase, and threonine deaminase, three of the enzymes participating in the biosynthesis of isoleucine and valine in the organism, and L-isoleucine inhibits the effect of valine (153). The inductive effect of L-valine appears to be due to its ability to inhibit the activity of acetohydroxy acid synthetase, thus causing isoleucine deficiency, which in turn leads to the derepression of the three enzymes.

Acetylated amino acids. Fowler, Camien, and Dunn (91) have identified acetyl L-isoleucine and acetyl L-leucine as extracellular products of *M. ranae*. The nature of the biosynthesis of these compounds would be most interesting, since acetylated amino acids have only rarely been encountered in natural materials, but when they do occur, as in mammalian systems, have been shown to possess profound physiological significance. Examples of the latter are acetyl-L-glutamic acid and acetyl-L-aspartic acid, intermediates in amino acid metabolism.

Paraaminobenzoic Acid

Sloane and co-workers (329–334) have studied in detail the metabolism of *p*-aminobenzoic acid (PABA). The washed cells of *M. smegmatis* form *p*-amino phenol from PABA as well as aniline. The first step in the sequence of metabolism of PABA is a direct enzymatic reduction of the carboxyl group to give *p*-aminobenzyl alcohol, which is followed by the transformation of *p*-aminobenzyl alcohol to *p*-hydroxy aniline. The authors have suggested that this unique transformation may be a specific case of a more general mechanism involved in enzymatic hydroxylation of aryl compounds. In the metabolism of aniline by mycobacteria also, aniline hydroxylation is preceded by hydroxymethylation. The sequence of reactions is:



However, *p*-aminobenzyl alcohol was not detected when ^{14}C-aniline was incubated with whole cells of *M. tuberculosis* in the presence or absence of ^{14}C-methyl methionine (230). When aniline is incubated with cell-free extracts, in the presence of labeled methionine, *N*-methyl aniline and small amounts of dimethylaniline can be isolated; methionine acts as the C-1 donor.

Carboligase

Yamasaki and Moriyama (426) have detected α-ketoglutarate carboligase activity in *M. phlei*. The reaction product, δ-(OH) levulinic acid, inhibits porphyrin synthesis from δ-amino levulinic acid in cell-free extracts of *M. phlei*, and therefore the carboligase may play an important part in the regulation of porphyrin synthesis (by inhibiting the δ-aminolevulinate dehydratase).

PROTEIN SYNTHESIS

The synthesis of proteins and nucleic acids by mycobacteria has attracted attention particularly from the view point of drug action (383, 384, 386, 387). While isoniazid inhibits the incorporation of α-ketoglutarate and DL-glutamate into proteins in *M. tuberculosis* H37Rv as well as *M. jucho* (an avirulent strain of *M. avium*), it does not inhibit the incorporation of glycine or leucine (386, 387).

The activation of various L-amino acids by cell-free extracts of *M. phlei* and *M. tuberculosis* H37Rv has been analyzed by the hydroxamate formation method (97, 98, 147). Cell-free extracts of *M. phlei* also activate D-amino acids such as D-glutamic acid or D-alanine, but these are not incorporated into transfer ribonucleic acid (tRNA). The activation of amino acids in nine different strains of mycobacteria is independent of the sensitivity or resistance of these organisms to streptomycin, isoniazid, *p*-amino salicylic acid, and ethionamide (146).

The incorporation of amino acids into proteins and the effect of antibiotics known to inhibit protein synthesis have been studied in mycobacteria also. The effect of streptomycin on the incorporation of ^{14}C-DL-tyrosine into proteins by cell-free extracts of *M. friburgensis*

was examined by Erdos and Ullmann (85) who claimed that streptomycin (100 μg/ml) inhibits the incorporation of tyrosine into proteins in streptomycin-sensitive cells but not in the streptomycin-resistant cells. However, the authors have employed the *p*H 4.5 precipitate obtained from the cell-free system prepared by centrifugation of extracts at 105,000 $\times$ *g* for 60 min and thus devoid of ribosomes. Later, these authors (86) noticed much lower inhibition of tyrosine incorporation in whole cells of *M. friburgensis* by streptomycin. Streptomycin causes almost complete inhibition of protein synthesis in whole cells of streptomycin-sensitive *M. tuberculosis* H37Rv but not in a streptomycin-resistant mutant (327*a*).

The amino acid incorporation by *M. smegmatis* 607 and BCG extracts is inhibited by chloramphenicol, tetracycline, erythromycin, and streptomycin (80). The steroidal antibiotics fusidic acid and helvolinic acid inhibit the incorporation of ^{14}C-leucine and ^{14}C-valine as well as polyuracil (poly U)-directed incorporation of phenylalanine in cell-free systems of *M. smegmatis* 607 (424).

Although Tomcsanyi (376) has described a system from *M. tuberculosis* H37Rv capable of incorporating labeled glycine, tyrosine, leucine, and valine in the absence of any added messenger, a systematic description of an amino acid incorporating system from mycobacteria was given for the first time by Rieber and Imaeda (309). They determined the optimal conditions for poly U-directed phenylalanine incorporation in the extracts of *M. smegmatis* 607 and demonstrated a slight stimulation of polyphenylalanine formation caused by isoniazid, presumably by enhancing the rate of activation of phenylalanine. An efficient system from *M. tuberculosis* H37Rv capable of incorporating phenylalanine directed by poly U has also been described recently (327*a*).

Ribosomes

Since mycobacteria are classified as *Actinomycetes* and are considered to be advanced prokaryotes, a look at the ribosomal structure to see whether they possess 70*S* ribosomes as the prokaryotes, or 80*S* ribosomes as the eukaryotes, should be fascinating. The isolation and characterization of ribosomes from *M. smegmatis* 607 and BCG have been reported by Eda (78, 79). The protein synthesizing activity of membrane-bound ribosomes from *M. smegmatis* 607 is higher than that of free ribosomes (93). Trnka, Wiegeshaus, and Smith (381) have characterized the ribosomes from BCG in greater detail. The BCG ribosomes sediment as a main peak of 70*S* and one peak each of 50*S* and 30*S* at an Mg^{2+} concentration of 10 mM. When the Mg^{2+} concentration is lowered to 0.1 mM, only 30*S* and 50*S* particles are observed, and the 70*S* particles completely disappeared. Under their experimental conditions, these authors could not detect any polysomes.

While analyzing the various cellular fractions of human tubercle bacilli for antigenic activity, Youmans and Youmans (431) described the preparation of ribosomes from *M. tuberculosis* H37Ra and demonstrated that the antigenicity was associated with the RNA components of these ribosomes. However, a detailed and systematic analysis of ribosomes from *M. tuberculosis* H37Ra was carried out by Worcel, Goldman, and Sachs (421). The H37Ra ribosomes also sediment as a 70*S* peak on sucrose gradients at 10 mM Mg^{2+}, whereas they dissociate into 50*S* and 30*S* particles when the Mg^{2+} concentration is lowered to 0.1 mM. Further, the RNA isolated from these ribosomes exhibits a sedimentation profile similar to the *Escherichia coli* ribosomal RNA, showing two major peaks of sedimentation, 23*S* and 16*S*, respectively, and a minor peak of 4 to 5*S*.

The structural integrity and functional capacity of the ribosomes, however, depend on the age of the culture and the method of preparation of bacterial extracts (378, 421). The ribosomal structures seem to disappear totally from old cells (4 weeks or older), and the protein synthesizing ability is also considerably less. In fact, Trnka and Smith (378) observed that poly U-dependent ^{14}C-phenylalanine incorporation by BCG ribosomes and supernatant fluids from 18-day-old cells is only 1.5 to 10% of the activity of 11-day-old cells, and 60 to 75% of the activity was lost by storing the extracts for 24 hr at 4 C. The system described by Trnka and Smith (378) is very efficient and gives an almost 100-fold stimulation of amino acid incorporation over background in response to the added messenger. Since several of the mycobacteria are slow-growing, a slower rate of protein synthesis was expected; however, the amount of RNA (about 26% of the dry weight) and proteins at the logarithmic phase of growth was approximately the same as found with bacteria having a faster rate of growth, although the amount of DNA (0.66% of dry weight) is lower than that found with most microorganisms (432).

NUCLEIC METABOLISM

General

The nucleic acids of tubercle bacilli attracted attention as early as 1898, and a product called "Tuberculinsaure" was isolated from "crushed tubercle bacilli" by Ruppel (314) which he described as possessing all the properties of a nucleic acid and also the "poisonous" and "immunizing" properties. The isolation and characterization of nucleic acids from *M. avium* (65), *M. phlei*, and *M. tuberculosis* (175, 220), and BCG (388) were described quite early. It is well known that mycobacterial DNA is characterized by an extremely high guanine-cytosine (GC) content, even though the significance of this is not known (64). The occurrence of modified bases such as 5-methyl cytosine and 6-methyl amino purine in mycobacterial nucleic acids was also established early (75, 174). In spite of all the early information available on the nucleic acids of mycobacteria, very little attention has been devoted to intermediary nucleic acid metabolism.

In the 1960's again several papers have appeared on the isolation of DNA from various species of mycobacteria and its GC contents. Among these, the only report of significance is from Wayne and Gross (396), who have described a technique for the isolation of DNA after autolysis of cells and have successfully applied this technique to eight species of *Mycobacteria*. Subsequently, these authors (128) using immobilized DNA (from various mycobacteria), labeled reference DNA (from *M. tuberculosis* and *M. kanasasii*), and annealing techniques compared the degrees of relatedness among various mycobacteria. Relative per cent binding and thermal stability of bound DNA are used as parameters for determining the homology; their results agree well with the established taxonomic data.

A gentle, but fast, method for isolating DNA from mycobacteria has been reported by Mizuguchi and Tokunaga (258) who lysed the cells with ethylenediaminetetraacetate and glycine or cycloserine before phenol extraction.

Recently Michalska and Lorenc (254) have described the isolation of nucleic acids from *M. smegmatis* 607 and BCG and their resolution on methylated albumin Kieselghur (MAK) columns into tRNA, DNA, and high-molecular-weight ribosomal RNA by salt gradient elution. However, this is only an extension of earlier work of Odrzywolska (274), who had examined the synthesis of RNA by labeling $^{32}P_i$ in normal cells of *M. smegmatis* 607 and in cells infected with phage D29 and had observed a rapidly labeled fraction, displaying a rapid turnover, presumably corresponding to the messenger RNA.

DNA and RNA

A report on the partial purification (430-fold) of DNA polymerase from *M. smegmatis* represents the only available information regarding the biosynthesis of DNA (415).

On growth under conditions of iron and zinc deficiency, an overall decrease has been observed in the synthesis of nucleotides or nucleic acid in *M. smegmatis* (137, 138, 139, 409, 416, 417). In a series of papers, Winder and coworkers (410–413) reported the presence and partial purification of the nucleoside triphosphate-dependent deoxyribonuclease in *M. smegmatis* and the characterization of the products of the enzyme reaction. There is a marked increase per unit of protein in the DNA polymerase and the ATP-dependent deoxyribonuclease activities, in iron-limited cultures, reaching the maximum between 48 and 72 hr of growth (414). The changes in activities of both enzymes can be prevented or reversed by addition of Fe^{2+} to normal values, within 24 hr. The two activities are apparently not the functions of one enzyme, since they have been separated [unpublished observations of F. G. Winder, M. Levin, and M. S. McNulty, cited by Winder and McNulty (415)].

The only enzyme pertaining to RNA metabolism in mycobacteria studied so far is polynucleotide phosphorylase. The enzyme has been reported to be present in *M. phlei* (57), *M. smegmatis* (59, 163), *M. avium* (14), and *M. tuberculosis* (250). Polynucleotide phosphorylase, partially purified from *M. tuberculosis* H37Rv, catalyzes the synthesis of polymers from nucleotide diphosphates, the phosphorylysis of polynucleotides, and the exchange reaction between nucleotide diphosphates and $^{32}P_i$. Further, the enzyme purified from H37Rv and H37Ra as well as from streptomycin-resistant (100 μg/ml) H37Rv cells exhibits very similar properties. The sensitivity to streptomycin of the enzymes derived from streptomycin-sensitive or streptomycin-resistant cells is the same; thus, the polynucleotide phosphorylase cannot be implicated as the locus of action of streptomycin.

Purines and Pyrimidines

The de novo biosynthesis of nucleic acid purines in *M. tuberculosis* H37Rv has been studied by using ^{14}C-labeled precursors, with special reference to the precursors of C_2 and C_8 of the purine ring by Malathi and Ramak-

rishnan (248). Both the ureide carbon atoms are derived most effectively from the β-carbon of serine and α-carbon of glycine, while formate, the "classical" precursor of the purine ring, is not an efficient C_1 donor in this organism. Cell-free extracts of the organism are also able to incorporate serine into the nucleic acid purines. Although ^{14}C-methyl-labeled methionine is not a precursor for the purines, the high specific radioactivity of the nucleic acids isolated from the organism grown in its presence suggests the methylation of bases in nucleic acid. Shaila and Ramakrishnan (328) have shown the presence of active tRNA-methylating enzymes in cell-free extracts of *M. tuberculosis* by using *S*-adenosyl methionine as methyl donor and methyl-deficient tRNA from *E. coli* as substrate.

Whole cells as well as cell-free extracts of *M. tuberculosis* H37Rv are capable of utilization and interconversion of preformed purines, adenine and guanine (247, 248). The conversion of guanine to adenine may be through the intermediate formation of guanosine monophosphate, inosine monophosphate, and AMP, and the deamination of guanine is ruled out. Mycobacteria can, however, deaminate adenosine (60), and some properties of adenosine deaminase from *M. tuberculosis* H37Ra have been described (134).

The ability of *M. butyricum* and *M. smegmatis* to oxidize uric acid to CO_2 and NH_3 with the intermediate formation of allantoin, allantoic acid, glyoxyl urea, and glyoxylic acid is reported by Klemperer, Scott, and Bagchi (198).

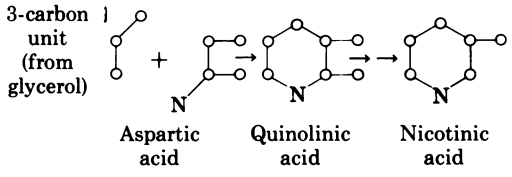
Very little information is available on the pyrimidine metabolism of mycobacteria. Recently, Vitol, Shaposhnikov, and Shvachkin (391) have reported that two strains of mycobacteria catabolize orotic acid to uracil, to barbituric acid, and to urea.

VITAMINS AND COENZYMES

Nicotinic Acid and Nicotinamide Coenzymes

The production of various B-complex vitamins by *M. tuberculosis* was analyzed by Pope and Smith (286) and Bird (36) and the synthesis of markedly high levels of nicotinic acid was observed. Later, Konno and his co-workers (205–207) made use of this excessive nicotinic acid production by *M. tuberculosis* as a method for differentiating the human strain of *M. tuberculosis* from other mycobacteria by employing a simple color reaction undergone by nicotinic acid with cyanogen bromide and aniline. Konno's experiments were repeated by several laboratories, and presently this method of differentiation has formed the basis of a simple biochemical test; however, each phase of Konno's original technique has undergone modification. In addition, these studies led to the search of other biochemical properties for the differentiation of tubercle bacilli.

A large number of workers have attempted to elucidate the mechanism of biosynthesis of nicotinic acid in mycobacteria (8, 125–127, 261, 264, 311). From both radioactive incorporation and nutritional supplementation studies, it has been shown that the biosynthetic mechanism in *M. tuberculosis* may be the following:



There is, however, no direct conversion of quinolinic acid to nicotinic acid.

A decrease in the production of nicotinic acid by BCG as a result of zinc deficiency or an increased supply of phosphate has also been reported (263, 435).

The biochemically functional forms of nicotinic acid are the nicotinamide nucleotide coenzymes (NAD and NADP), which are synthesized by way of nicotinic acid mononucleotide (NaMN) and nicotinic acid adenine dinucleotide (NaAD). The direct conversion of quinolinate to NaMN without the intermediary formation of free nicotinic acid by the enzyme quinolinate transphosphoribosylase has been reported for various strains of mycobacteria, including *M. tuberculosis* H37Rv and H37Ra (209, 210). This is in contrast with the results of Dudley and Willet (73, 74) who reported the conversion of nicotinic acid to NaMN in *M. bovis* and *M. tuberculosis*. However, earlier studies of Gopinathan (119) had demonstrated the inability of H37Rv extracts to incorporate ^{14}C-labeled nicotinic acid or nicotinamide into NaMN and NaAD, in agreement with the results of Konno et al. (210). Thus the excretion of nicotinic acid may represent the inability of the organism to utilize this compound for the biosynthesis of the coenzymes. Under conditions of ATP limitation, quinolinate transphosphoribosylase can degrade the NaMN formed into free nicotinic acid (258), and the enzyme may thus possess regulatory properties.

The degradation of NAD (or NADP) is accomplished by the enzyme NADase (NAD glycohydrolase) which cleaves the nicotinamide-riboside bond of the oxidized form of the coenzymes. This enzyme can, in addition to the hydrolytic cleavage, catalyze an exchange reaction between NAD and free nicotinamide or compounds structurally related to it, resulting in the formation of an analogue of NAD (433). The potent antitubercular drug isoniazid can participate in the NADase-catalyzed exchange reaction, and the isoniazid analogue of NAD formed may not be effective as the coenzyme. This was suggested as the mechanism of action of isoniazid, and therefore the enzyme is of great significance in mycobacteria.

NADases have been reported in *M. butyricum* (189, 371) and *M. tuberculosis* H37Rv (121, 122), and in both the cases the enzymes are associated with a heat-labile protein inhibitor. The partially purified enzymes from both of these strains do not catalyze the NAD-isoniazid exchange reaction. The proteinaceous inhibitor of NADase from *M. tuberculosis* H37Rv has also been purified and its specificity as well as certain aspects of enzyme-inhibitor complex formation were evaluated (120). The inhibitor is specific for mycobacterial NADase and the enzyme-inhibitor complex formed is undissociable. The complex formation between highly purified NADase (molecular weight, 39,000) and its proteinaceous inhibitor (molecular weight, 26,000) from *M. butyricum* has recently been studied by Ogasawara et al. (275) by use of Sephadex G-100 chromatography, sucrose gradient centrifugations, and cellulose acetate electrophoresis.

The NADase inhibitor may play a role in the regulation of NAD levels and seems to be sensitive to the action of isoniazid. The latter property will be discussed in the section of mechanism of drug action.

The presence of active NADase in lung-grown tubercle bacilli was shown by Artman and Bekierkunst (20); it is presumably derived from the infected tissues. The tremendous increase in NADase activity in tuberculous tissues has also been shown to be of host tissue origin and not derived by activation of bacterial enzyme (123).

The nicotinamide, rising out of NADase action, can be deamidated to nicotinic acid by an enzyme nicotinamidase, which is also present in several strains of mycobacteria, with the exception of *M. bovis* (208). More details about the enzyme are given in another section of this review.

Other B Vitamins

Compared to the volume of literature available on nicotinic acid and its derivatives, the information available on other vitamins and coenzymes in mycobacteria is scanty.

Distribution of thiamine and its mono-, di-, and triphospho derivatives was quantitatively determined in *M. lacticola* and *M. fortuiitum* by Rossi-Fanelli et al. (313). All of the thiamine esters can be dephosphorylated to thiamine in cell-free extracts of *M. lacticola*.

The effects of medium composition (C, N, P, Fe, K, Mn, and surface active agents) and growth conditions (the intensity of aeration, temperature, and *p*H of the medium) on the yield of flavine from *M. smegmatis* strain 104 were studied by Mil'ko and Ivanova (256). Flavine synthesis increases with increasing concentrations of Na and K, but increasing concentration of PO_4 decreases it; low initial *p*H of the medium favors flavine biosynthesis.

The synthesis of dihydrofolate and dihydropteroate from dihydroneopterin by extracts of *M. smegmatis* has been reported by Jaenicke et al. (168). The isomers of the pteridine precursors are neither substrates nor inhibitors of folate biosynthesis.

The synthesis of vitamin B_{12} by various strains of mycobacteria was examined by Aithal and Sirsi (2), who found some differences in the nature and contents of B_{12} in isoniazid-sensitive and -resistant strains of mycobacteria, assayed microbiologically with *Lactobacillus leichmannii* 313. The significance of these results, however, is not established.

Vitamin K

Among the fat-soluble vitamins, only the K-group of vitamins from mycobacteria has received attention; their role in electron transport has been well documented and has already been considered under the section of electron transport. The natural naphthoquinone (dihydromenaquinone-9) has been resolved into two geometric isomers, and the *cis* isomer is active in oxidative phosphorylation (76). The report that *M. phlei* also contains dihydromenaquinone-8, and probably dihydromenaquinone-10, shows the necessity for detailed studies on the role of these quinones, if any, in oxidative phosphorylation (58).

Attempts have been made to establish the biosynthesis of vitamin K in mycobacteria. [2-^{14}C]Mevalonic acid and acetate-2-^{14}C are not incorporated into vitamin K_2 in *M. tubercu-*

losis or *M. phlei* (301). The incorporation of radioactivity from methionine-*methyl*-^{14}C into the 2-methyl group of vitamin K_2-(45)-H by whole cells as well as cell-free extracts of *M. phlei* has been reported by Azerad, Bleiler-Hill, and Lederer (30) and Guerin, Azerad, and Lederer (131). Under their conditions, labeled tyrosine-*U*-^{14}C is not incorporated into vitamin K_2. Recently Azerad and co-workers (132, 228) have presented some evidence for the biosynthesis of the ring of naphthoquinone in *M. phlei* and *M. avium* derived from studies of the incorporation of labeled substrates into the vitamin. While *p*-hydroxybenzaldehyde and 3,4-dihydroxybenzaldehyde are not incorporated, [*U*-^{14}C]shikimic acid served as good precursor of the napthoquinone rings. Tentative schemes for naphthoquinone ring biosynthesis involving *O*-carboxyphenylpyruvic acid as an intermediate have been suggested but have yet to be confirmed. According to the authors, menadione may not be a true intermediate in menaquinone biosynthesis. Incorporation of α-naphthol-*1*-^{14}C into naphthoquinones has also been examined; while one strain of *M. phlei* (Legroux) shows a fair amount of incorporation of radioactivity, only a small amount is incorporated by another strain, *M. phlei* (ATCC 354).

MISCELLANEOUS ENZYME SYSTEMS

A number of unrelated enzymes in mycobacteria have been reported, and for the most part these have been used to differentiate mycobacterial species and strains from one another. The role of most of these enzymes in the overall metabolism of mycobacteria is not clear. The enzymes include phosphatases (15, 269, 317, 363, 389), nicotinamidase and formidase (129, 136, 189–194, 208, 266), urease (18, 286), arylsulfatase (285, 360–362), reductase (359, 366, 382), esterases (28, 395), and deaminase (434); special mention should be made about catalase and peroxidase in mycobacteria. Mycobacteria are catalase-positive organisms and also show peroxidative activity towards certain phenols like pyrogallol. Both activities are lacking in isoniazid-resistant strains of *M. tuberculosis* (255). There has been a controversy whether catalatic and peroxidatic activities of these organisms are due to one and the same enzyme or to two distinct enzymes. In support of the second hypothesis it has been reported (368, 369) that some species of mycobacteria and some strains of *M. tuberculosis* are negative to a qualitative test for peroxidatic activity but give a positive catalase test. Further, the two activities from certain strains of *M. avium* are separated by zone electrophoresis at *p*H 8.6 on cellulose acetate (16). On the other hand, Winder (404) has presented evidence that peroxidatic activity in *M. smegmatis* is due to catalase since, during 60-fold purification of catalase, all fractions showed the same ratio of catalase to peroxidase activity as the crude extracts. Similar experiments carried out with *M. tuberculosis* and other mycobacteria may clear up the controversy.

MECHANISM OF ACTION OF ANTITUBERCULAR DRUGS

Isoniazid

The drugs currently in use against tuberculosis are isoniazid, streptomycin, *p*-aminosalicylic acid (PAS), cycloserine, ethambutol, rifamycin, pyrazinamide, ethionamide, kanamycin, capreomycin, viomycin, and thioacetazone. An exhaustive account of all the antitubercular drugs known so far is given by Robson and Sullivan (312). Isoniazid is the most potent antitubercular drug available and is specific in its action against mycobacteria. A voluminous amount of literature has accumulated on the mode of action of this drug and the subject has been reviewed periodically (113, 405, 428). Some of the earlier hypotheses put forth to explain the action of isoniazid are: (i) the drug inhibits the synthesis or activity of hemoproteins like catalases or peroxidase; (ii) isoniazid is active because of its chelating property; (iii) hydrogen peroxide and free radicals formed during the metabolism of the drug are toxic to *M. tuberculosis;* (iv) the drug acts through its metabolic products; (v) the drug acts as a respiratory inhibitor; (vi) the drug interferes with pyridoxal and nicotinic acid function; (vii) the drug inhibits the aspartic-glutamic transaminase of the organism; and (viii) the drug interferes with the carbohydrate, lipid, protein, and nucleic acid metabolism of the organism. We shall deal in this article mostly with literature which has appeared after the last review by Youatt (428) was published.

Any hypothesis proposed to explain the action of isoniazid should account for the following facts: (i) the drug is specific in its action against mycobacteria, and (ii) the growth-inhibitory concentration of the drug is very low. Two hypotheses which apparently satisfied these conditions at the time the last review appeared are the following. (i) Isoniazid

combines with an enzyme that is specific to the drug-susceptible strains of *M. tuberculosis*, modifying the enzymes and at the same time displacing a molecule which gives rise to a pigment precursor. Further reactions of either the pigment precursor or the modified enzyme in the presence of appropriate substrates lead to derangement of the various metabolic activities of the organism. Youatt and Tham (429) have shown that this enzyme reaction has many features in common with the growth-inhibiting action of isoniazid. Further, the reaction is not observed in isoniazid-resistant cells. The biochemical characteristics of this enzyme system from *M. tuberculosis* H37Rv have been described recently from the authors' laboratory (103*a*). (ii) Isoniazid combines with the heat-labile inhibitor of NADase present in *M. tuberculosis* and this causes a decrease in the content of NAD. The lowering of NAD content affects the metabolic activities of the organism. In support of this hypothesis, Bekierkunst and Bricker (5) reported that in an isoniazid-resistant mutant of *M. tuberculosis* H37Ra the inhibitor of NADase is altered in such a way that it is not inactivated by isoniazid. The resistant organism they used is a multiple-step mutant, and it is not clear therefore that the observed reaction is a result of the primary action of the drug or one of the several changes which has occurred. To clarify the situation, Sriprakash and Ramakrishnan (340) isolated a number of independent mutants of *M. tuberculosis* H37Rv which were exposed to the lowest inhibitory concentration of isoniazid and hence would reasonably be altered in only one biochemical step. These independent mutants can be divided into two groups—one in which the inhibitor has lost its sensitivity to the drug, and the other where the inhibitor is unchanged. In both groups, the uptake of ^{14}C-labeled isoniazid was very much decreased as compared to the sensitive organism, but when the permeability barrier to the drug was removed by the use of surface-active agents, the drug proved to be lethal to the organism. Even though the NADase inhibitor has lost its sensitivity to isoniazid in one group of isoniazid-resistant mutants, not only does the drug exert its lethal activity against these mutants (in the presence of surface-active agents), but it also decreases their NAD concentration, as in the case of the drug-sensitive parents (338). There seems to be, therefore, a direct correlation between the lethality of isoniazid and the lowering of NAD content; since isoniazid cannot bind to the NADase inhibitor in this case, nor does it directly activate the NADase of *M. tuberculosis* (122), the mechanism by which the drug brings about the decrease of NAD in the organism is unknown. However, isoniazid at a concentration of 0.5 mM inhibits the synthesis of NAD by cell-free extracts of *M. tuberculosis* H37Rv and the isoniazid-resistant mutants to the same extent (339).

One has to conclude that up to the present time no satisfactory hypothesis about the mode of action of isoniazid is available. Recently, Winder and Collins (408) have suggested that the primary site for the inhibitory action of isoniazid lies in the biosynthetic pathways to the mycolic acids. The inhibition of mycolic acid synthesis may lead to the formation of defective envelope material low in mycolic acid, resulting in loss of materials from the cell leading ultimately to loss of acid fastness and death. The model is yet to be proved experimentally.

Other Antitubercular Agents

Although the mechanism of action of streptomycin is now well established in *E. coli*, very few reports on its mode of action on mycobacteria are available. In view of the reports that the lipid content is doubled in a streptomycin-resistant strain of *M. tuberculosis* H37Rv as compared to a sensitive strain (63), that the drug inhibits arginase in whole cells of *M. smegmatis* but not in cell-free extracts (270), and that it induces premature lysis of phage-infected mycobacteria (374), a systematic examination of the mode of action of streptomycin in mycobacteria seems to be desirable.

Hedgecock (140–143) demonstrated that strains of *M. kansasii* have more resistance to PAS in vitro than *M. tuberculosis* and that partial reversal of PAS inhibition occurs in the presence of methionine only for the former organisms. The fact that *M. kansasii* can utilize the free methionine present in human serum, while *M. tuberculosis* cannot, probably explains why PAS is ineffective as a chemotherapeutic agent against *M. kansasii*.

Among rifamycins, rifamycin SV and rifampin inhibit at low concentrations the growth of *M. tuberculosis* and other mycobacteria. White, Lancini, and Silvestri have demonstrated (401) that the primary event in the inhibition of *M. smegmatis* by rifampin is the block of DNA-dependent RNA polymerase, just as in *E. coli*. Since, however, the effect of rifampin on mycobacteria is slower than on *E. coli* the authors conjecture that there might be a difference in the stability of the rifampin-RNA polymerase complex between those bac-

teria which are killed very rapidly and those which are killed more slowly. The other reported effects of rifampin on the incorporation of ^{32}P into the nucleic acids of *M. tuberculosis* (384) and on the poly U-dependent phenylalanine incorporation by isolated ribosomes of mycobacteria (379) are probably only secondary.

D-Cycloserine is employed as a second-line chemotherapeutic agent against *M. tuberculosis*. D-Alanine antagonizes the inhibition of growth of *M., tuberculosis* and *M. phlei* by cycloserine, while L-alanine competes in the protection given by D-alanine (262, 436). D-Alanine is a constituent of the mycobacterial cell wall, and in the presence of D-cycloserine a mucopeptide accumulates in the drug-sensitive strain of *M. acapulcensis*.

David, Takayama, and Goldman (69, 70) have isolated D-alanyl-D-alanine synthetase from *M. tuberculosis* H37Ra and shown that the kinetics of the inhibition of this enzyme by D-cycloserine are similar to that from *Staphylococcus aureus* and *Streptococcus faecalis*, which are less sensitive to D-cycloserine. On the other hand, these authors have shown that the synthesis of Wax D peptidoglycolipid of the H37Ra strain is inhibited by D-cycloserine at low concentration and that this inhibition is completely reversed by D-alanine. An arabinogalactan-galactosamine-(diaminopimelic acid)-mycolate accumulates in the organism exposed to the antibiotic, and the authors suggested that this lipid is a possible precursor to the synthesis of Wax D. Further work on this system may reveal the particular enzyme which is inhibited by the drug and the role played by D-alanine in the reaction.

D-Carbamyl serine is another antibiotic which has antitubercular action. Its mode of action in *S. faecalis* has been reported to be through its inhibition of alanine racemase, thus depriving the organism of D-alanine necessary for cell wall biosynthesis (241). The basis of its antitubercular action, however, appears to be different. The inhibitory effect of D-carbamyl serine in *M. tuberculosis* H37Rv is not reversed by L-alanine or D-alanine unless the bacilli are previously grown in media supplemented by D- or L-alanine, suggesting that there is an inducible alanine permease system in the organism (68). The drug potentiates the inhibitory action of D-cycloserine, and the potentiation is partially reversed by D-alanine. The author has concluded without any additional direct evidence that D-carbamyl serine inhibits the induction of an alanine permease system.

Ethambutol has specific antimycobacterial activity and has been shown to be therapeutically effective in tuberculosis in experimental animals and in man. Even though the drug is taken up rapidly by both proliferating and nonproliferating *M. smegmatis*, it has no effect on the viability and metabolism of nonproliferating cells (89, 90, 214). The synthesis of DNA and protein and, to a lesser extent, RNA, is inhibited in the presence of the drug. Polyamines and magnesium added to the culture reverse the inhibitory action of the drug. Since spermidine and magnesium are necessary for maximal synthesis of RNA, ethambutol may be exerting its inhibitory effect by interfering with a function of polyamines and cations in nucleic acid metabolism. Whether these effects are due to the primary action of the drug or are secondary effects is not clear. A more extensive investigation of the mode of action of ethambutol in mycobacteria is therefore called for.

Bacitracin at low concentrations inhibits the growth of *M. smegmatis* and, at higher concentrations, the growth of BCG (310). Ultrastructure studies indicated that the main target of bacitracin on mycobacteria may be the membrane.

From the above account it may be seen that the mode of action of practically none of the currently available drugs on mycobacteria has been satisfactorily explained and remains even today a challenge to intending investigators in the area.

GENETICS OF MYCOBACTERIA

General

A beginning has been made in recent years to study the genetics of mycobacteria. The results of such studies would be of valuable assistance in relating the data on metabolism to the genetic constitution of mycobacteria and to understand the problem of drug resistance and virulence. Of the genetic processes reported to be operative in mycobacteria, only lysogenic conversion and transduction are reasonably well documented. A large number of bacteriophages of mycobacteria has been isolated; the work carried out on these has been reviewed by Redmond (304). These phages have been mostly used for typing mycobacteria.

Lysogenic Conversion

The mycobacteriophage B2 lysogenizes the host strain *M. phlei* F89 and on reisolation it shows a genotype which differs in several re-

spects from B2; previously restricted to *M. phlei* F89, its host range now extends to *M. smegmatis* SN2 (179). This phage was called B2h. Since no absorption studies of the phages B2 and B2h with the two bacterial strains have been reported, it is difficult to judge whether the ability of B2h to plaque on *M. smegmatis* SN2 is due to an extension of the host range of the phage or due to the ability of B2h to adsorb on to SN2. Concurrently with the changes in phage type, the respective lysogenic hosts show an altered colony morphology and a decreased growth rate, and the authors refer to this as lysogenic conversion. When phage B2h is grown on *M. smegmatis* SN2, a fraction of the infected cells gives rise to typical *M. phlei* colonies (177). In similar experiments with B2h grown on *M. phlei* F89, conversion of *M. phlei* to *M. smegmatis* was found. The conversion is mediated by the hybrid phage B2h, which is able to lysogenize both *M. phlei* and *M. smegmatis*. One interpretation from these results for the difference between *M. phlei* and *M. smegmatis* chromosomes is that an addition of a specific region to *M. smegmatis* chromosomes results in the *M. phlei* chromosome, and a deletion of this region from the *M. phlei* chromosome gives rise to *M. smegmatis* chromosome. B2h could mediate this process (directly or indirectly).

In another set of experiments, Juhasz (178) studied the changes in *M. phlei* due to B2h lysogeny by using the replica plating method. The changes could be divided into three categories: conversion to slow growth and small colony size, dependence on related but complex nutritional requirements, and conversion to thiamine auxotrophy.

The infection of *M. smegmatis* 607 by phages D29 and B4 results in colony change from rough to smooth and increased nitrate reductase activity accompanying lysogenization (176). Loss of the lysogenic state reverses these changes. It has been suggested that the observed rapid loss and gain of such characters are due to genetic changes brought by a plasmid. Since lysogens of *E. coli* which contain defective prophages have been reported to form wrinkled colonies (233, 234), this explanation for the colony change is probably the most logical one. Juhasz, Gelbart, and Harize (180) have reported decrease in the amidase, nitrate reductase, and phenolphthalein sulfatase activities of *M. smegmatis* SN2 following lysogenization by phage B1, showing that a wide range of changes may follow lysogenization and alter potentially important taxonomic characters. An extreme case of such changes has been found in *Mycobacterium* strain 80 which is obtained following lysogenization of *M. phlei* F89 with phage B2h (105). The per cent GC content (68.9) of the DNA of strain 80 is intermediate between that of *M. phlei* (69.9) and *M. smegmatis* (67.9). The authors admit that a 1% difference may not be considered significant but add that another F89 (B2h) lysogen which does not show any of the changes as strain 80 has the same GC content (69.9) as the parental *M. phlei*.

Some of the earlier investigators had reported the isolation of smooth colonies of mycobacteria from previously rough strains after exposure to mycobacteriophages (316, 400). Russell, Jann, and Froman (316) have also demonstrated that some smooth colonies were lysogenized with the infecting phage. Because of the low frequency with which smooth colonies are detected and the inability to demonstrate lysogeny in many of the smooth colonies, they believed that selection of spontaneous mutants is the preferable explanation for their findings. White, Foster, and Lyon (400), however, isolated smooth colonies of *M. smegmatis* 607 after exposure to certain phages in a higher frequency than would be explained by selection of mutants alone. The high frequency with which smooth colonies are demonstrated to be lysogenic suggests that lysogeny is responsible for the altered colony morphology. Thus, reports of lysogenic conversion in mycobacterium have appeared from different laboratories, but the spectrum of genetic characters altered in the lysogenic conversions studied by Bonicke and Juhasz is much broader than those studied by others and requires careful analysis for any possible artifact brought about by the problem of clumping in mycobacteria. Interconversion of species mediated by phage, if confirmed, would have far-reaching genetic and taxonomic implications, especially in light of the hypothesis that the atypical mycobacteria have originated from the tubercle bacilli "through genetic mechanisms" (152).

Transduction

A mycobacteriophage capable of mediating transduction in *M. smegmatis* SN2 has been isolated recently by Sundar Raj and Ramakrishnan (345). Transduction has been shown for histidine, glycine, arginine, alanine, and adenine markers; the transduction of resistance to the drug isoniazid (to which only mycobacteria are susceptible) can now be attempted. The authors have also described methods of isolating auxotrophs of *M. smeg-*

matis by using ultraviolet irradiation and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NG) as mutagens (346). The use of NG, hydroxylamine, ultraviolet irradiation, and ethylmethane sulfonate for isolating mutants of *M. phlei* (auxotrophic, drug-resistant, as well as mutants with changed pigmentation) have been described by Konickova-Radochova and co-workers (201, 204). An interesting observation they have reported is that among the auxotrophic mutants, 50% required either glycine or serine for growth. Transduction in *M. phlei* by another mycobacteriophage, Bo2, has been recently reported (105*a*).

Transfection

Tokunaga and Nakamura (372, 373) have successfully infected *M. tuberculosis* and *M. smegmatis* in the late log phase of growth with DNA extracted from mycobacteriophage B1. One microgram of DNA (the equivalent of 1.5×10^8 phage) produced 84 infective centers. The activity was destroyed by deoxyribonuclease but not by phage antiserum. Transfectibility was increased by presensitization of bacteria to cycloserine and amino acids such as D-serine, D-alanine, D-lysine, D-threonine, and glycine, apparently by alteration of the cell surface (375). *M. smegmatis* 607 becomes highly competent for transfection when incubated in the standard acid medium, which is a modification of the minimal medium for mycobacteria (267).

Conjugation

Mizuguchi and Tokunaga (259) recently reported genetic recombination between auxotrophs of *M. jucho* and *M. lacticola* in solid media at a frequency of about 10^{-3} to 10^{-5}. Crosses between mutants of homologous strains were not fertile, nor were recombinants produced in liquid broth. Pretreatment of either of the parental types with acridines did not abolish the ability to produce recombinants. This observation eliminated the possibility that the donor in the recombination corresponded to an F^+ mating type, but the use of very few auxotrophic markers in the recombination experiments makes it difficult to judge whether the donor corresponded to the Hfr type. It is hoped that with the isolation of a greater variety of auxotrophic mutants not only will this problem be solved but also that attempts will be made to map the mycobacterial chromosomes. Mycobacterial conjugation has sexual polarity and in a study of the kinetics of gene transfer, the glycine marker entered earlier than the leucine marker (Tokunaga and Mizuguchi, *personal communication*). This observation supports the idea that an Hfr mediates the conjugation.

Mapping

A partial map of the genome of mycobacteriophage D29 by the isolation of temperature-sensitive mutants and the determination of recombinational sequences has been constructed by Mizuguchi and Sellers (260). By growing the mutants at the permissive temperature for varying intervals and then subjecting them to the restrictive temperature they were able to find out the physiological role of the various genes, as has been done in T4 phages.

CONCLUDING REMARKS

The information presented here shows that a significant amount of work on the metabolism of mycobacteria has been carried out since the last review was published. However, some metabolic problems have received little attention, while others seem to have been overemphasized.

The metabolism of carbohydrates and lipids by mycobacteria has been extensively studied, and the operation of classical pathways such as the glycolytic pathway, hexose monophosphate shunt, citric acid cycle, and β-oxidation has been well documented. Several enzymes of these pathways have been partially purified, but the lactate oxidative decarboxylase of *M. phlei* and *M. smegmatis* is the only mycobacterial enzyme that has been crystallized. Again a comparison of oxidative and glycolytic pathways in virulent and avirulent strains of mycobacteria has been attempted. This attempt was made in order to discover whether the capacity of the virulent strains to multiply in the host tissues is due to differences in oxidative metabolism or not; it is known that the oxygen tension in the host tissues is low. Because the differences between virulent and avirulent strains seem to be quantitative, no definitive explanation has been possible.

Some of the metabolic properties of tubercle bacilli grown in vivo (in susceptible laboratory animals) and in vitro (in chemically defined media) have been compared, and some interesting differences have been found. More detailed studies on such differences would be desirable.

Electron transport and oxidative phosphorylation in mycobacteria, particularly in *M. phlei*, have been studied thoroughly by Brodie and his colleagues. The phosphorylation sites

and the coupling factors of the respiratory chain have been characterized. Limited information is available on *M. tuberculosis* also, but the pathogenic strains of *M. tuberculosis* need systematic investigation.

Some headway has been made on problems of the biosynthesis of simple fatty acids in mycobacteria. Our knowledge of the biosynthesis of more complex fatty acids and lipids is still based on data obtained from whole cells by using some labeled precursors, or from cell-free systems in which only a few steps have been worked out. Detailed studies with purified enzymes or enzyme complexes of lipid biosynthesis and the determination of cofactor requirements of the enzymes should be undertaken.

There are certain qualitative differences in the amino acid metabolism of the virulent (H37Rv) and the avirulent (H37Ra) strains of *M. tuberculosis*. The enzyme asparaginase, responsible for the breakdown of asparagine (which is the preferred nitrogen source for their growth in synthetic media), is distributed differently in the virulent and avirulent strains. While the H37Rv strain possesses only one type of asparaginase, the H37Ra strain possesses two asparaginases. Notably, the enzyme specific to H37Ra is inducible; it inhibits the growth of Yoshida ascites sarcoma in rats, and its inhibitory activity is superior to that of other bacterial asparaginases studied hitherto. Yet another difference between the two strains is that the avirulent strain possesses a specific aspartotransferase, which transfers the aspartyl moiety of asparagine to hydroxylamine, and the avirulent strain does not. The possible role of these enzymes in the phenomenon of virulence is an open question.

Studies on nucleic acids and protein synthesis of mycobacteria are still at the preliminary stage; such attempts have been largely restricted to studies on the incorporation of simple labeled precursors into these macromolecules and the effects of antitubercular drugs on precursor incorporation. A systematic approach to this problem is urgently needed.

The study of the genetics of mycobacteria is still in its infancy. Successful use of various chemical mutagens for the isolation of auxotrophic and other mutants of mycobacteria has been reported, but here again peculiar problems are posed because of the tendency of mycobacteria to extreme clumping and their relatively long generation time. The former problem, however, may be solved by isolating mutants which do not clump. Various bacteriophages specific for mycobacteria have been isolated, including some capable of transducing genetic markers. The existence of different mating types in mycobacteria has also been reported recently. These observations raise hopes for unveiling the genetic make-up of the mycobacterial chromosome. Although successful transfection has been carried out with isolated phage DNA, all attempts to demonstrate transformation in mycobacteria have failed. The fragmentary genetic information on mycobacteria available at present is restricted to the nonpathogenic, relatively fast-growing species.

Knowledge on the mode of action of various antitubercular drugs also is still scanty. Most of the research on the action of drugs has been done at the level of substrate utilization, or by testing them on isolated enzymes. Hence, these studies fail to explain the specificity of the drug or the development of drug resistance. Earlier work showed that the development of resistance to the drug isoniazid is accompanied by a loss of catalase or peroxidase activity of mycobacteria; this finding suggests that the study of metabolic differences, as a consequence of the development of drug resistance, could lead to fruitful results.

The information currently available on the metabolic pathways in mycobacteria is not sufficient for devising new chemotherapeutic drugs against mycobacteria. However, a potential method for developing such drugs could probably be based on the biosynthetic pathways unique to these organisms, such as those for vitamin K and mycobactins. A drug which inhibits any of the enzymatic steps in these pathways is likely to prevent the growth of an organism like *M. tuberculosis* without in any way harming the human host.

Except for a few reports on the phenolases and glutamate decarboxylase, nothing is known about the metabolic activities of the important species, *M. leprae*. The development of suitable culture media for the in vitro growth of such organisms would greatly stimulate research in this important area.

ACKNOWLEDGMENTS

The work described in this paper from the authors' laboratory was supported by grants from the Indian Institute of Science, the Indian Council of Medical Research, and the Bhabha Atomic Research Centre. Most of the equipment used in the work has been provided by grants from the Rockefeller Foundation, New York, and from the Wellcome Trust, London. The preparation of this article is supported by the Indian Institute of Science.

The authors are also grateful to several of their colleagues and in particular to Barry Bean, C. K. R. Kurup, and P. S. Sastry for reading, criticizing, and discussing the manuscript.

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